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COMPOSITIONS AND METHODS UTILIZING SEQUENCES FOR CONTROLLING NUCLEIC ACID EXPRESSION IN YEAST

BACKGROUND OF THE INVENTION

The controlled production in yeast of an enormous variety of useful proteins or polypeptides can be achieved using recombinant DNA technology. Yeast cells can be transformed with yeast expression vectors, which contain homologous or heterologous nucleic acid molecules encoding polypeptides (coding sequences). The yeast cells can then produce large quantities of the useful proteins or polypeptides in yeast cell culture.

Expression of the nucleic acid molecule encoding a polypeptide by the yeast expression vector is initiated at a region known as the promoter, which is recognized by and bound by RNA polymerase. The RNA polymerase travels along the DNA, transcribing the information contained in the coding strand from its 5' to 3' end into messenger RNA, which is in turn translated into a polypeptide having the amino acid sequence for which the DNA codes. The present invention provides novel yeast promoters useful for, *inter alia*, controlling the expression of homologous and heterologous nucleic acid sequences encoding proteins and polypeptides in yeast cells.

SUMMARY OF THE INVENTION

It is an object of the invention to provide novel yeast promoters, yeast expression vectors, and transformed yeast cells. It is a further object of the invention to provide a method for producing proteins and polypeptides in yeast cell culture.

In one embodiment of the invention a yeast promoter which comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide is provided. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

(54) Title: COMPOSITIONS AND METHODS UTILIZING SEQUENCES FOR CONTROLLING NUCLEIC ACID EXPRESSION IN YEAST

(57) Abstract: The invention provides novel yeast promoters useful for controlling the expression of homologous and heterologous nucleic acid molecules in yeast cells. The yeast promoters are induced by a fermentable carbon source, such as glucose, or a non-fermentable carbon source, such as ethanol, or both. Therefore, expression of nucleic acid molecules encoding a polypeptide under the control of the novel yeast promoters may be regulated by varying the level of a fermentable carbon source, or a non-fermentable carbon source, or both.

The promoter is operative when operably linked to a nucleic acid molecule encoding a polypeptide.

As used herein, the term *Apromoter* refers to a nucleic acid sequence which is capable of initiating transcription of a nucleic acid molecule encoding a polypeptide (coding sequence); a *Yeast promoter* is capable of initiating transcript of a coding sequence in yeast cells; and *Apromoter activity* refers to the level or amount of transcription initiation of a coding sequence, and encompasses any level above background (*i.e.*, the level or amount that occurs in the absence of a promoter; a background level, which is normally zero).

Another embodiment of the invention provides a yeast promoter which comprises an isolated and purified polynucleotide. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4. The promoter is operative when operably linked to a nucleic acid molecule encoding a polypeptide.

Yet another embodiment of the invention provides a yeast promoter fragment which comprises at least 17 contiguous nucleotides of a polynucleotide. The polynucleotides are shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4. The fragment has promoter activity as determined by cloning the fragment into a yeast expression vector, wherein the fragment is operably linked to a reporter gene, transforming yeast cells with the yeast expression vector, growing the yeast cells in yeast cell culture under conditions favorable for expression of the reporter gene, and assaying the yeast culture for a reporter protein expressed by the reporter gene. The expression of the reporter gene indicates the fragment has promoter activity.

Still another embodiment of the invention provides a yeast expression vector comprising a yeast promoter. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4. The promoter is operative when operably linked to a nucleic acid molecule encoding a polypeptide.

A further embodiment of the invention provides a yeast expression vector where activity of the promoter is controlled by varying the level of a non-fermentable carbon source, such as ethanol, in a medium of yeast cells in culture. The yeast cells are transformed with said yeast expression vector.

In yet another embodiment of the invention, a yeast expression vector comprising a yeast promoter which comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide is provided. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4. Promoter activity is controlled by varying the level of a fermentable carbon source in a medium of yeast cells in culture, where the yeast cells are transformed with the yeast expression vector. The fermentable carbon source can be glucose.

Another embodiment of the invention provides a yeast expression vector comprising a yeast promoter. The yeast promoter comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4. Promoter activity is controlled by varying the level of a fermentable carbon source and a non-fermentable carbon source, such as ethanol, in a medium of yeast cells in culture, where the yeast cells are transformed with the yeast

expression vector. The fermentable carbon source can be glucose. The non-fermentable carbon source can be ethanol.

Still another embodiment of the invention provides a yeast cell transformed with a yeast expression vector. The yeast expression vector comprises a yeast promoter. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4. The promoter is operative when operably linked to a nucleic acid molecule encoding a polypeptide.

Yet another embodiment of the invention provides a method for producing a polypeptide. A yeast expression vector is constructed where a polynucleotide encoding the polypeptide is controlled by a yeast promoter. The yeast promoter comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4. The promoter is operative when operably linked to a nucleic acid molecule encoding a polypeptide. A culture of yeast cells is transformed with the yeast expression vector. The yeast cells are maintained in culture so that the polypeptide is expressed. The polypeptide is then recovered.

Still another embodiment of the invention provides a method for producing a polypeptide. A nucleic acid molecule encoding the polypeptide is cloned into an expression vector selected from the group consisting of pYLR110P+luc, pYMR251AP+luc, pYMR107P+luc, pZEO1P+luc, pYLR110P, pYMR251AP, pYMR107P, and pZEO1P. The nucleotide acid molecule is operably linked to a promoter of the expression vector. A culture

of yeast cells is transformed with the yeast expression vector. The yeast cells are maintained in culture so that the polypeptide is expressed and the polypeptide is then recovered.

Another embodiment of the invention provides a method for producing a polypeptide. A yeast expression vector is constructed where a nucleic acid molecule encoding the polypeptide is controlled by a yeast promoter. The yeast promoter comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4. Yeast cells are transformed with the yeast expression vector and are maintained in culture medium. The expression of the nucleic acid molecule encoding the polypeptide is controlled by varying the level of a fermentable carbon source, such as glucose, in the culture medium. The polypeptide is then recovered.

Still another embodiment of the invention provides a method for producing a polypeptide. A yeast expression vector is constructed where a nucleic acid molecule encoding the polypeptide is controlled by a yeast promoter. The yeast promoter comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4. The promoter is operative when operably linked to a nucleic acid molecule. A culture of yeast cells is transformed with the yeast expression vector. The yeast cells are maintained in culture medium and the expression of the nucleic acid molecule encoding the polypeptide is controlled by varying the level of a non-fermentable carbon source, such as ethanol, in the culture medium. The polypeptide is then recovered.

Another embodiment of the invention provides a method for producing a polypeptide. A yeast expression vector is constructed where a nucleic acid molecule encoding the polypeptide is controlled by a yeast promoter. The yeast promoter comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide. The promoter sequences are shown in SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4. A culture of yeast cells is transformed with the yeast expression vector. The yeast cells are maintained in culture medium and the expression of the nucleic acid encoding the polypeptide is controlled by varying the level of a fermentable carbon source, such as glucose, and a non-fermentable carbon source, such as ethanol, in the culture medium. The polypeptide is then recovered.

Yet another embodiment of the invention provides a method of identifying a promoter fragment with promoter activity by generating a fragment comprising at least 17 contiguous nucleotides of an isolated and purified polynucleotide. The polynucleotides are shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4. The fragment is cloned into a yeast expression vector, so that the fragment is operably linked to a reporter gene. Yeast cells are transformed with the yeast expression vector and grown in yeast cell culture under conditions favorable for expression of the reporter gene. The yeast culture is assayed for a reporter protein expressed by the reporter gene. Expression of the reporter gene indicates the fragment has promoter activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a map of YEpl3 expression vector.

Figure 2 schematically illustrates construction of YLR110C and YMR251WA promoter constructs.

Figure 3 is a map of pPRB1P.

Figure 4 is a map of pPRB1P+luc.

Figure 5 is a map of pYLR110P+luc.

Figure 6 is a map of pYMR251AP+luc.

Figure 7 is a map of pYMR107P+luc.

Figure 8 is a map of pZEO1P+luc.

Figure 9 is a map of pYLR110P.

Figure 10 is a map of pYMR251AP.

Figure 11 is a map of pYMR107P.

Figure 12 is a map of pZEO1P.

Figure 13 schematically illustrates the YLR110C promoter region.

Figure 14 schematically illustrates the YMR251WA promoter region.

Figure 15 schematically illustrates the YMR107W promoter region.

Figure 16 schematically illustrates the ZEO1 promoter region.

DETAILED DESCRIPTION OF THE INVENTION

Novel yeast promoters whose activity can be controlled by a fermentable carbon source, such as glucose, or a non-fermentable carbon source, such as ethanol, or both have been identified. The yeast promoters are useful for, *inter alia*, the high level production of proteins or polypeptides in yeast cell culture.

Yeast Promoters

The isolated and purified promoter polynucleotides of the invention are shown in SEQ ID NO:1 (the YLR110C promoter), SEQ ID NO:2 (the YMR251WA promoter), SEQ ID NO:3 (the YMR107W promoter), and SEQ ID NO:4 (the ZEO1 promoter). Yeast promoters comprising as little as 17 nucleic acids have been determined to function as promoters. The yeast promoters of the invention comprise at least 17, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600 or 700 contiguous nucleic acids of an isolated and purified polynucleotide up to the maximum length provided in any one of the sequences presented herein, that is, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

Preferably, the promoter polynucleotides are isolated free of other components, such as proteins and lipids. The polynucleotides can be made by a cell and isolated or can be synthesized in the laboratory, for example, using an automatic synthesizer or an amplification method such as PCR.

Naturally occurring variants and artificial sequence variants (that is, those which do not occur in nature) of the promoters are included in the invention. Variants of the promoters and/or fragments thereof have, along their entire length, sequence identity of at least 90%,

and preferably greater than 95% as determined by the Smith-Waterman homology search algorithm as implemented in MPsrch™ program (University of Edinburgh) using an affine gap search with the following search parameters: gap open penalty: 12, gap extension penalty: 1.

Fragments of the full-length promoters are also functional as promoters. A promoter fragment of at least 17 contiguous nucleotides may occur at any position along the full-length promoter as shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4. Accordingly, promoter activity of 17 or more contiguous nucleotides occurring anywhere along the full-length promoter can be analyzed. Fragments of 17, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650 or 700, nucleotides of the promoters may be constructed by, for example, subjecting an isolated promoter to restriction endonucleases, to 5'- or 3'-deletion mutagenesis, to PCR, or to site specific deletion. A combination of these methods can also be used to generate fragments of a promoter.

The invention further embodies a hybrid promoter, *i.e.*, a promoter that comprises more than one promoter or more than one fragment of a promoter from which it was derived.

The promoter fragments can be derived from more than one of the promoter sequences shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4. The promoters and fragments can be constructed as described above, ligated together, and cloned into a yeast expression vector. Where a promoter comprises nucleotides from at least two polynucleotides selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4, at least 5, 6, 7, 8, 9, 10, 25, 50, 75, 100, 150, 200, 250, 300, 350,

400, 450, 500, 550, 600, or 650 contiguous nucleotides are derived from each of the polynucleotides to form a promoter of at least 17 nucleotides. Alternatively, each of the full-length promoters can be combined with another full-length promoter or with fragments of another promoter.

The yeast promoters, fragments of the promoters, and hybrid promoters are useful for controlling expression of a protein or polypeptide when the yeast promoter is operably linked to a nucleic acid molecule encoding the protein or polypeptide.

Determination of Promoter Activity

Promoters and fragments of promoters can be assayed for promoter activity by cloning a fragment of a promoter, or a full-length promoter, or a hybrid promoter into a yeast expression vector so that is operably linked to a reporter gene, *i.e.*, a coding sequence for a reporter protein. The yeast expression vector is transformed in yeast cells, which are grown in yeast cell culture under conditions favorable for expression of the reporter gene, for example, under conditions providing a fermentable and/or non-fermentable carbon source. Expression of the reporter gene, as determined by an assay for the amount of a reporter protein expressed by the reporter gene, indicates that the promoter has activity.

For example, to determine if a promoter has activity, *i.e.*, is operative, expression of a reporter gene by a promoter of the invention may be compared to expression of the reporter gene by a reference promoter such as PBR1 (Cottingham *et al.* (1991) *Eur J Biochem* 196(2):431-8; Sleep *et al.* (1991) *Biotechnology* 9(2):183-7; Finnis *et al.* (1992) *Yeast* 8(1):57-60; Meldgaard *et al.* (1995) *Glycoconj J* 12(3):380-90; Bach *et al.* (1996) *Receptors*

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and Channels 4(2):129-39. A promoter, a fragment of a promoter, or a hybrid promoter of the invention is operative if it expresses at least 25% of the amount of a reporter protein as the full-length PBR1 promoter in a medium containing a non-fermentable carbon source, or a fermentable carbon source, or both. Preferably, an operative promoter expresses at least 50%, 75%, 100%, 200%, 300%, 400%, or more of the amount of a reporter protein as the full-length PBR1 reference promoter.

Assays for promoter activity are useful for identifying yeast promoters with high activity and the specific nucleotide sequences of the promoters that are necessary for promoter activity.

Yeast Expression Vectors

The yeast promoters of the invention, which comprise isolated and purified polynucleotides selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4 or fragments thereof, can be used to construct yeast expression vectors.

Yeast expression vectors are any vectors capable of autonomous replication within a yeast host organism or capable of integrating into the yeast genome. Yeast expression vectors are useful for introducing foreign DNA into yeast cells. Typical yeast expression vectors include yeast integrative plasmids (YIp), yeast replicating plasmids (YRp), yeast expression plasmids (YXp), yeast centromere-containing plasmids (YCp), and yeast episomal plasmids (YEp). Preferably, a yeast expression vector can be selected and maintained in both yeast and *E. coli*.

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Yeast expression vectors, typically plasmids, incorporate the yeast promoters of the invention to control expression of nucleic acid molecules encoding heterologous or homologous proteins or polypeptides. The nucleic acid molecules are operably linked to a promoter in the yeast expression vector. A wide range of heterologous eukaryotic and prokaryotic proteins or peptides may be expressed by the vectors of the invention.

Expression vectors incorporating the promoters can be constructed by inserting into a vector a nucleic acid molecule encoding a protein or polypeptide (coding sequence) which is to be expressed. The coding sequence can be inserted at a restriction site which is provided downstream of a translation start codon controlled by the promoter. The coding sequence must be inserted in the correct translational reading frame.

Alternatively, the polynucleotide can itself be provided with a translational start codon followed directly by a coding sequence. Where the promoter does not contain a translational start codon, a restriction site is provided so that the coding sequence can be inserted in the correct reading frame and so that its translational start codon is correctly positioned in relation to the promoter. The coding sequence can encode heterologous or homologous or eukaryotic or prokaryotic polypeptides or proteins. In a preferred embodiment the coding sequence encodes a fusion protein. The coding sequence may further comprise a signal sequence.

In addition to the promoters of the invention, other components can be added to the expression vectors of the invention. For example, yeast selective markers, such as *LEU2* or *TRP1*, which allow for selection of yeast cells that have been effectively transformed by the

vector can be added. A yeast replication origin, such as the replication origin of the 2-micron plasmid or the autonomous ARS replication segment can be added. Upstream activating sequences and transcription terminator sequences may be added. Further, at least a portion of a bacterial plasmid, such as found in YEpl3, can be added to enable the yeast expression vector to be manipulated in an intermediate bacterial host system, such as *Escherichia coli*.

The expression vector may also comprise a reporter gene which encodes, for example, β -galactosidase or luciferase. The reporter gene can be under the control of a promoter of the invention. Where the reporter gene, *i.e.*, coding sequence, is linked to a gene encoding a desired protein, assaying the level of expression of the reporter protein can quickly and easily determine the level of expression of the desired protein.

The expression vectors of the invention can be used to direct the fermentable carbon source- and/or non-fermentable carbon source-induced high level expression of proteins or polypeptides in yeast. The promoters of the invention can be induced by the presence of a fermentable carbon source, such as glucose, or a non-fermentable carbon source, such as ethanol, or both. That is, the promoters have greater promoter activity in the presence of a fermentable carbon source, or a non-fermentable carbon source, or both than in the absence of a fermentable carbon source, or a non-fermentable carbon source, or both. Promoters

YLR110C, as shown in SEQ ID NO:1; YMR251WA, as shown in SEQ ID NO:2; and ZEO1, as shown in SEQ ID NO:4, can be induced by a fermentable carbon source, such as glucose, or by a non-fermentable carbon source, such as ethanol, or by both. Promoter YMR107W, as shown in SEQ ID NO:3, can be induced by a non-fermentable carbon source, such as

ethanol. Thus, the amount of expression of a homologous or heterologous nucleic acid molecule encoding a protein operably linked to the promoters of the invention can be controlled by varying the amount of an available fermentable carbon source, such as glucose, or a non-fermentable carbon source, such as ethanol, or both.

5 Transformed Yeast Cells

Yeast cells can be transformed with the yeast expression vectors of the invention.

Transformation can be accomplished by well known methods, including, but not limited to electroporation, calcium phosphate precipitation, and microinjection. The yeast expression vectors of the invention can be used to transform yeast cells, including, but not limited to *Saccharomyces cerevisiae*, *S. uvarum*, *S. carlsbergensis*, *Saccharomycopsis lipolytica*, *Schizosaccharomyces pombe*, and *Kluyveromyces fragilis*.

Transformed yeast cells containing a yeast expression vector can be grown in an appropriate medium for the yeast. A fermentable or non-fermentable carbon source can be added to the yeast culture medium in order to control the activity of the promoter.

15 Methods of Production of Proteins

Yeast cells transformed with expression vectors comprising a promoter of the invention can be used to produce proteins and polypeptides. Under proper cell culture conditions, preferably in the presence of a fermentable or non-fermentable carbon source, or both, the promoters of the invention will control expression of a nucleic acid molecule encoding a polypeptide operably linked to the promoter.

The protein or polypeptide can be retained within the yeast cell. The yeast cells can be then harvested, lysed, and the protein obtained and substantially purified in accordance with conventional techniques. Such techniques include, but are not limited to chromatography, electrophoresis, extraction, and density gradient centrifugation.

5 In a preferred embodiment of the invention, the protein or polypeptide to be recovered will further comprise a signal peptide capable of transporting the protein or polypeptide through the membrane of a transformed yeast cell. The protein or polypeptide can be recovered from the culture medium by, for example, adsorption or precipitation.

Further, the proteins and polypeptides may be produced as a fusion protein, which includes not only the amino acid sequence of the desired protein, but also one or more additional proteins. Affinity purification protocols can be used to facilitate the isolation of fusion proteins. Typically, a ligand capable of binding with high specificity to an affinity matrix is chosen as the fusion partner for the desired protein. For example, fusion proteins made with glutathione-S-transferase can be selectively recovered on glutathione-agarose and IgG-Sepharose can be used to affinity purify fusion proteins containing staphylococcal protein A.

Preferably, the protein or polypeptide of interest can be separated from the remainder of the fusion protein. The fusion protein can be constructed so that a site for proteolytic or chemical cleavage is inserted between the protein of interest and the fusion partner. For example, sites for cleavage by collagenase, Factor Xa protease, thrombin, and enterokinase, have been inserted between the fusion partner and the protein of interest. The protein of

interest can be also cleaved from the remainder of the fusion protein by chemical cleavage by, for example, hydroxylamine, cyanogen bromide (CNBr), or N-chlorosuccinimide.

The following are provided for exemplification purposes only and are not intended to limit the scope of the invention described in broad terms above. All references cited in this disclosure are incorporated by reference.

EXAMPLE 1

Preparation of Yeast Samples

S. cerevisiae strain 11C

This example describes the growth of haploid *Saccharomyces cerevisiae* strain 11C. It has the genotype: *ade2-161, trp1-Δ63, ura3-52, lys2-801, leu2Δ1* &/or *leu2-112, his3Δ200* &/or *his4-519*. 11C was generated by crossing the strains YPH500 (Mat a *ura3-52 lys2-801 ade2-161 trp1-Δ63 his3Δ200 leu2Δ1*) (Sikorski and Hieter. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122: 19-27) and AH22 (MATa *leu2-3 leu2-112 his4-519*) (Hinnen *et al.* (1978) Transformation of yeast. *Proc. Natl. Acad. Sci. USA* 75: 1929-1933).

Three sterile 500 ml conical flasks, each containing 100 ml sterile YPD broth (Sigma, Cat No. Y-1375) were inoculated with sterile 10μl loops of differing quantities of the *S. cerevisiae* strain 11C from a freshly streaked YPD plate (Sigma, Cat No. Y-1500), and grown in an orbital shaker at 30°C, 200 rpm, overnight. The growth of 11C in the three flasks was measured by absorbance at 600nm. One flask was deemed to be at the late

exponential growth phase (1.98 ODU ml at 600 nm), and this culture was used to inoculate (50ml o/n culture per flask) 2 identical 5L sterile conical flasks (labeled E and L), each containing 1L sterile YPD broth to a final concentration of ~0.1 ODU ml. Flasks E and L were grown in an orbital shaker at 30°C, 200 rpm. 10ml samples were collected at times indicated below (Table 1). The samples were treated as follows: their growth was determined (A600nm), the possibility of contamination was checked (using a light microscope), cells were harvested in a benchtop centrifuge (~2000xg for 5 minutes), and the supernatant removed and frozen at -20°C (samples labeled E0 - E3, and L0 - L5).

Table 1. *Growth of cultures E and L as measure by absorbance at 600 nm.*

Time Point	Time after inoculation (min)	Growth of flask E (ODU)	Growth of flask L (ODU)
T0	0	0.099	0.099
T1	310	0.37	0.36
T2	410	0.71	0.72
T3	455	0.97	0.92
T4	775	-	3.64
T5	1420	-	6.05

After 455 minutes, a time deemed to be late exponential growth phase in glucose, flask E (*i.e.* early) was harvested (~2000xg for 5 minutes), split into 50ml aliquots, and frozen at -80°C. After 1420 minutes, a time deemed to be growth on ethanol, flask L (*i.e.* late) was harvested (~2000xg for 5 minutes), split into 50ml aliquots, and frozen at -80°C.

Determination of Glucose and Ethanol concentration

Supernatant samples (E0 - E3, and L0 - L5) were defrosted, and their ethanol and glucose contents were measured using ethanol (Boehringer, Cat. No. 176290) and glucose (Boehringer, Cat. No. 176251) detection kits according to manufacturers instructions. The concentrations determined are shown below in Table 2.

Table 2. Glucose and Ethanol concentrations in supernatants of cultures E and L at different time points.

Sample	Time after inoculation (min)	Glucose level in media (g L ⁻¹)	Ethanol level in media (g L ⁻¹)
E0	0	20.0	0.0
E1	310	21.8	0.3
E2	410	21.8	0.8
E3	455	21.2	0.87
L0	0	20.0	0.0
L1	310	22.2	0.36
L2	410	22.0	0.62
L3	455	20.0	0.87
L4	775	11.8	5.2
L5	1420	0.0	11.8

It can seen in Table 2 that at the point of culture harvest for E (E3, 455 minutes) the cells were still utilizing glucose as a carbon source, while at the point of culture harvest for L (L5, 1420 minutes) glucose was exhausted, and the cells were utilizing ethanol as a carbon source. Calibration values used to calculate glucose concentrations are shown in Table 3. Calibration values used to calculate ethanol concentrations are shown in Table 4.

Table 3. Glucose standards

GLUCOSE STANDARDS g/L	OD A340
0	0
0.2	0.246
0.4	0.461
0.6	0.726
0.8	0.967
1	1.227

Table 4. Ethanol standards

ETHANOL STANDARDS g/L	OD A340
4.72	0.041
9.44	0.083
18.88	0.166
37.76	0.322
56.6	0.534
75.5	0.664
94.4	0.846

Analysis of RNA Levels From Yeast Dimorphic Growth Samples

Total RNA Isolation

Total RNA was isolated from 300ml of culture using the hot phenol protocol. The frozen yeast pellets were resuspended in lysis buffer (4ml) (0.5ml Tris-CL (1M, pH 7.5), 1.0 ml EDTA (0.5 M), 2.5ml 10% SDS, and 46.0ml ddH₂O) and an equal volume of acid phenol

was added and vortexed. Following incubation at 65°C for one hour (with occasional vigorous vortexing) the mixture was placed on ice for 10 minutes then centrifuged (10 minutes). The aqueous layer was transferred to a fresh centrifuge tube and mixed with an equal volume of phenol at room temperature. The mixture was centrifuged and an equal volume of chloroform was mixed with the aqueous layer in a fresh centrifuge tube. Following centrifugation the aqueous layer was transferred to a fresh centrifuge tube and sodium acetate (to a final concentration of 0.3M) and two volumes of 100% ethanol was added to precipitate the RNA. The mixture was placed at -20°C for 30 minutes then centrifuged for 10 minutes to pellet the RNA. The RNA pellet was washed 2-3 times with 70% ethanol then allowed to dry at room temperature. The pellet was resuspended in ddH₂O (200-500 µL). The RNA was quantitated by measuring OD 260-280. Yield of total RNA was ~4.5mg from each culture.

Poly A+ RNA Purification

Poly A+ RNA was purified from total RNA using Qiagen Oligotex mRNA Midi Kit (Qiagen, Cat. No. 70042). 2mg of total RNA was used as starting material and made up to a volume of 500µl with DEPC treated H₂O. To this 500µl buffer OBB (2x binding buffer) and 55µl oligotex suspension was added. The A Oligotex mRNA Spin-Column Protocol® from the kit protocol booklet was followed. The pelleted mRNA was washed in 200µl 75% ethanol, dried and resuspended in 10µl DEPC treated H₂O. Yield of Poly A+ RNA was ~8µg for each sample.

cDNA Synthesis

cDNA was synthesized using the protocol for GeneChip Expression Analysis Manual using reagents from Gibco BRL Life Technologies Superscript Choice System cat. No. 18090-019. For each sample 5µg Poly A+ RNA was added to 100pmol of T7-(dT)₂₄ primer (sequence: GGCCAGTGAATTGTAATACGACTCACTATAGGGAGCGG-(T)₂₄, HPLC purified) (SEQ ID NO:15) in a total of 8µl (made up to volume with DEPC treated H₂O). The reaction mixture was incubated for 10 minutes at 70°C in a Perkin Elmer PE9600 thermocycler then put on ice. The following reagents were added to the reaction mixture: 4µl 5x first strand cDNA buffer; 2µl 0.1M DTT; and 1µl 10mM dNTP mix. The reaction mixture was mixed and incubated at 37°C for 2 minutes in a Perkin Elmer PE9600 thermocycler. 5µl SuperScript II reverse transcriptase was then added. The mixture was incubated at 37°C for 1 hour in a Perkin Elmer PE9600 thermocycler.

The first strand cDNA reaction was placed on ice and the following reagents added: 9µl DEPC treated H₂O; 30µl 5x second strand reaction buffer; 3µl 10mM dNTP mix; 1µl 10units/µl *E. coli* DNA ligase; 4µl 10units/µl *E. coli* DNA Polymerase I; and 1µl 2units/µl RNase H. The mixture was incubated at 16°C for 2 hours in a Perkin Elmer PE9600 thermocycler. 2µl 5units/µl T4 DNA Polymerase was then added. The mixture was incubated for a further 5 minutes at 16°C in a Perkin Elmer PE9600 thermocycler. 10µl 0.5M EDTA was then added.

The double stranded DNA was cleaned up by phenol extraction. The reaction product transferred to a 1.5ml eppendorf tube and 162µl Tris pH 8.0 saturated phenol was

added. The tube was mixed by vortexing, the tube was then centrifuged in a microfuge at 13,000rpm for 5 minutes. The top fraction was recovered and cDNA precipitated by addition of 60µl 7.5M ammonium acetate plus 400µl absolute ethanol. This was immediately centrifuged in a microfuge at 13,000rpm for 20 minutes. The supernatant fraction was discarded, the pellet was washed in 75% ethanol and then air-dried. The pellet was resuspended in 20µl DEPC treated H₂O

Synthesis of Biotin-Labeled cRNA by In Vitro Transcription (IVT)

Reagents from Ambion MEGAscript T7 kit, cat. No. 1334, were used for the synthesis of biotin-labeled cRNA by *in vitro* transcription (IVT). The NTP Labeling mix comprised 7.5mM ATP; 7.5mM GTP; 5.625mM UTP; 1.875mM Biotin-16-UTP (Enzo cat No. 42814); 5.625mM CTP; and 1.875mM Biotin-11-CTP (Enzo cat No. 42818). The IVT Labeling reaction comprised: 14.5µl NTP Labeling mix; 2µl 10x Ambion Transcription Buffer; 1.5µl Double strand cDNA (from above); and 2µl Ambion T7 Enzyme Mix.

The reaction mixture was incubated for 6 hours at 37°C in a Perkin Elmer PE9600 thermalcycler. The biotinylated cRNA was cleaned up using Qiagen RNeasy kit, cat No. 74103. The RNeasy kit protocol was followed exactly. RNA was eluted in 2 aliquots of 30µl DEPC treated H₂O. The RNA was precipitated by addition of 6µl 3M sodium acetate pH 5.5 plus 75µl absolute ethanol. The RNA was allowed to precipitate overnight at -20°C. Samples were centrifuged in a microfuge at 13,000rpm for 20 minutes to pellet the RNA. The supernatant fraction was discarded and the pellet was washed in 1ml of 75% ethanol

and then allowed to air dry. The pellet was then resuspended in 20µl DEPC treated H₂O. The yield of cRNA was ~ 40µg for each sample.

cRNA Fragmentation

1µg of cRNA was fragmented. 8µl of 5x Fragmentation buffer (200mM Tris-Acetate pH 8.1, 500mM potassium acetate, 150mM magnesium acetate) plus 11µg cRNA made up to 20µl with DEPC treated H₂O was used. The reaction mixture was incubated 94°C for 35 minutes in a Perkin Elmer PE9600 thermal cycler.

Hybridization to GeneChip Microarray

The hybridization mix comprised: 20µl (11µg) of fragmented cRNA; 2.2µl of control oligo B2 (50pmol/µl) (sequence: 5= Biotin-GTCAAGATGCTACCGTTACAG 3= HPLC purified) (SEQ ID NO:16); 2.2µl Herring Sperm DNA (10mg/ml); 110µl 2x Buffer (2M NaCl, 20mM Tris pH 7.6, 0.01% Triton X-100); and 85.6µl DEPC treated H₂O. The hybridization mix heated to 95°C in a Techne hot block for 5 minutes, followed by incubation at 40°C for 5 minutes. The hybridization mix was clarified by centrifugation in microfuge at 13,000rpm for 5 minutes.

200µl of supernatant to added to the GeneChip cartridge (GeneChip cartridge was previously pre-wetted with 200µl 1x Buffer and incubated for 10 minutes at 40°C in the rotisserie box of a GeneChip hybridization oven 320 (cat No. 800127) at maximum rpm. The sample was hybridized to the microarray overnight at 40°C in a GeneChip hybridization oven in the rotisserie at maximum rpm.

Washing and Staining of Probe Arrays

The hybridization mix was recovered from the GeneChip cartridge and put back in the tube containing the remainder of the sample. 200µl 6x SSPE-T (6x SSPE plus 0.005% Triton X-100) was applied to the chip and pipetted in and out twice. This process was repeated twice more. Another 200µl 6x SSPE-T was applied to the cartridge and the cartridge was then incubated for 1 hour at 50°C at maximum rpm in the GeneChip hybridization oven. The 6x SSPE-T was removed and 200µl 0.5x SSPE-T was added to the cartridge. The cartridge was incubated for 15 minutes at 50°C at maximum rpm in the GeneChip hybridization oven. The 0.5x SSPE-T was removed and the cartridge was re-filled with 200µl 6x SSPE-T.

10 The stain solution comprised: 190µl 6x SSPE-T; 10µl of 20mg/ml acetylated BSA; and 2µl 1mg/ml conjugated streptavidin:phycoerythrin (Molecular Probes cat. No. S-866). 200µl 6x SSPE-T was removed from the GeneChip cartridge and 200µl of stain solution added. The cartridge was incubated at ambient temperature in a GeneChip hybridization oven at maximum rpm in the rotisserie for 10 minutes. The stain solution was removed and the cartridge was washed by adding 200µl 6x SSPE-T and pipetting this in and out of the cartridge twice. This process was repeated six times. The cartridges were then completely filled with 6x SSPE-T and any bubbles removed. Hybridization, washing and staining was repeated using the same hybridization mixes until both samples had been hybridized to each of the four yeast chip sub-set arrays.

20 Data collection

Data was collected by scanning the hybridized chips on a Hewlett-Packard GeneArray scanner. A AhaloØ effect (appearance of stain non-specifically across the array image) was seen on one of the scanned images: yeast growing in glucose rich media, sub-set C array. Scanning of this array was aborted after one scan and the chip was washed twice with 200µl 6x SSPE-T and then re-filled as before. This array was then re-scanned three times and the data collected was the average of these three scans. All other arrays were scanned four times without problems and the data collected was the average of the four scans.

EXAMPLE 3

10 Isolation of promoters and construction of expression vectors.

PCR amplification of promoter regions from genomic DNA

Based on the *Saccharomyces cerevisiae* genomic sequence in the GenEMBL nucleotide database oligonucleotide primers were designed to amplify the genomic sequence 5= to the following ORFs: YLR110C (Johnston *et al.* (1997) Nature 1997 May 29;387(6632 Suppl):87-90), YMR251WA (common name HOR7) (Bowman *et al.* (1997) Nature May 29;387(6632 Suppl):90-3), YMR107W (Bowman *et al.* (1997) Nature May 29;387(6632 Suppl):90-3), and YOL109W (common name ZEO1) (Dujon *et al.* (1997) Nature May 29;387(6632 Suppl):98-102). The region amplified was the non-coding region separating the selected ORF and the next predicted *Saccharomyces cerevisiae* ORF in the 5= direction, with a minimum length of 500bp.

Sequence of oligonucleotide primers used to amplify promoter DNA

HindIII, NdeI cloning sites underlined.

5	YLR110C-F	ATCGAAGCTTCGGCGCCGCCCTGATTTCCGTTT	SEQ ID NO:5
	YLR110C-R	CCAGCGCGCATATGTCATATAGTGTTAAG	SEQ ID NO:6
	YMR251W-A-F	AGCTAAGCTTCGGCGCCGCCCTTTCGATTACACGCAC	SEQ ID NO:7
	YMR251W-A-R	AGATACCTTGATATGTTATTAATAGTC	SEQ ID NO:8
10	YMR107W-F	AGCTAAGCTTCGGCGCCGCCGAGAAATGATGAAG	SEQ ID NO:9
	YMR107W-R	ATCCATCCCATATGTTGATATCTCGATTAG	SEQ ID NO:10
	ZEO1-F	AGCTAAGCTTCGGCGCCGCCGAGGTCTCTTCACG	SEQ ID NO:11
15	ZEO1-R	TACGATCGCATATGTAATTGATATAAAG	SEQ ID NO:12

PCR reactions were set up for each primer pair as follows: For YMR251W-A and ZEO1 90µl of Reddy-Load PCR (1.1X) mix, 3.5mM MgCl₂ (Advanced Biotechnologies, cat.no. AB-0628); 2µl of forward primer (100µM); 2µl of reverse primer (100µM); 1µl of *S. cerevisiae* genomic DNA (Promega G310A, lot 8347702, 276µg/ml); and 5µl of H₂O were combined.

For YLR110C and YMR107W 90µl of Reddy-Load PCR (1.1X) mix, 1.5mM MgCl₂ (Advanced Biotechnologies, cat.no. AB-0575); 2µl of forward primer (100µM); 2µl of reverse primer (100µM); 1µl of *S. cerevisiae* genomic DNA (Promega G310A, lot 8347702, 276µg/ml); and 5µl of H₂O were combined.

The thermocycling was carried out as follows: For the YMR251W-A promoter: 94°C for 5 minutes followed by 30 cycles of : 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute; followed by 72°C for 5 minutes. The reaction mixtures were then held at 4°C. For the YMR107W and ZEO1 promoters: 94°C for 5 minutes followed by 30 cycles of : 94°C for 30 seconds, 45°C for 30 seconds, 72°C for 1 minute; followed by 72°C for 5 minutes.

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The reaction mixtures were then held at 4°C. For the YLR110C promoter: 94°C for 5 minutes followed by 30 cycles of : 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute; followed by 72°C for 5 minutes. The reaction mixtures were then held at 4°C.

The PCR solutions were loaded onto an LMP gel and the bands were purified using

Wizard PCR Preps (Promega, cat. no. A7170) according to protocol, eluted in 50µl ethanol precipitated, and resuspended in 20µl. A map of the YLR110C promoter region is shown in Figure 13 and SEQ ID NO:29. A map of the YMR251W-A promoter region is shown in Figure 14 and SEQ ID NO:30. A map of the YMR107W promoter region is shown in Figure 15 and SEQ ID NO:31. A map of the ZEO1 promoter region is shown in Figure 16 and SEQ ID NO:32.

Cloning promoter regions into a yeast vector containing the luciferase gene

The PCR products representing the regions upstream of the YLR110C and YMR251W-A ORFs were cloned into the suitably digested YEp13-based multicopy yeast expression vector pPRB1P+Luc. A map of YEp13 is shown in Figure 1. The Accession number for YEp13 is U03498. A map of pPRB1P is shown in Figure 2. The sequence of pPRB1P is shown in SEQ ID NO:27. A map of pPRB1P+Luc is shown in Figure 3 and the sequence is shown in SEQ ID NO:28. The PRB1 promoter was removed from the vector by digesting with the restriction enzymes HindIII and NdeI. The digested backbone was then ligated with a HindIII / NdeI digested PCR product. See Figure 4.

The PCR products described below, and maxi-prepped pPRB1P+Luc were digested as follows. 60 µl of pPRB1P+Luc (328µg/ml), 10 µl of Hind III (Life Technologies, cat.no.

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15207-012, 10 units/ μ l), 10 μ l NdeI (Amersham, cat.no. E0216Y, 20 units/ μ l), 10 μ l NEBuffer 2 (NEB, cat.no. 007-2), and 10 μ l of H₂O. 14 μ l YLR110C, 2 μ l of Hind III (Life Technologies, cat.no. 15207-012, 10 units/ μ l), 2 μ l Nde I (Amersham, cat.no. E0216Y, 20 units/ μ l), and 2 μ l NEBuffer 2 (NEB, cat.no. 007-2). 14 μ l YMR251WA, 2 μ l of Hind III (Life Technologies, cat.no. 15207-012, 10 units/ μ l), 2 μ l Nde I (Amersham, cat.no. E0216Y, 20 units/ μ l), and 2 μ l NEBuffer 2 (NEB, cat.no. 007-2). The solutions were allowed to react at 37°C, for 4 hours.

The double digested pPRB1P+luc backbone was purified on an LMP gel using Wizard PCR preps (Promega, cat. no. A7170), and then ethanol precipitated. The remaining digestion products were also ethanol precipitated. The pPRB1P+luc digests were resuspended in 60 μ l of H₂O and the PCR product digests were resuspended in 20 μ l.

Ligation reactions were then carried out between each promoter region and the digested pPRB1P+luc at 16°C overnight. The PCR products representing the regions upstream of the following ORFs: YMR107W and ZEO1, were prepared, restricted, and ligated essentially as described above, however BCL restriction buffer B and different amounts of PCR product/volumes were used.

Transformation of ligation products into *E. coli*

The products of the ligations described above were transformed into *E. coli* (Invitrogen=s One-Shot TOP10 Competent cells, cat.no. C4040-10) according to manufacturers protocol. In each case 5 μ l of the ligation product was added to the cell

suspension. The total final cell suspension was plated out onto L-amp plates and incubated overnight at 37°C.

Colonies were picked from the plates and PCR screened using the PCR primers used to amplify the promoters originally. Two positive colonies from each ligation were grown in 5ml overnight cultures and their plasmids were purified (Promega Wizard Plus SV Minipreps, cat. no. A1330). The eluted DNA was ethanol precipitated and resuspended in 20 μ l of water. Analytical restriction digests were carried out to confirm the presence of the correct promoter. Clones containing all four promoter constructs were obtained.

The new constructs were named as follows:

- 10 pPRB1+luc backbone + YLR110C promoter = pYLR110P+luc SEQ ID NO:19
- pPRB1+luc backbone + YMR251WA promoter = pYMR251AP+luc SEQ ID NO:20
- pPRB1+luc backbone + YMR107W promoter = pYMR107P+luc SEQ ID NO:21
- pPRB1+luc backbone + ZEO1 promoter = pZEO1P+luc SEQ ID NO:22

- 15 Maps of pYLR110P+luc, pYMR251AP+luc, pYMR107P+luc, and pZEO1P+luc are shown in Figures 5, 6, 7, and 8, respectively. Plasmid DNA (pYLR110P+luc and pYMR251AP+luc) was prepared for transformation into yeast and sequencing using the QIAGEN Plasmid Maxi kit (Cat.no. 12162). The DNA concentrations of the maxi-preps (measured by absorbance at 260 nm) were: pYLR110P+luc 463 μ g/ml; pYMR251AP+luc 346 μ g/ml; pYMR107P+luc ~300 μ g/ml; and pZEO1P+luc ~720 μ g/ml. The remaining plasmids were transformed into yeast as Wizard Plus SV Mini-prep DNA, and maxi-prep DNA was obtained for sequencing using the Gibco BRL Concert Plasmid Maxi kit (Cat no.11452).
- 20

Sequencing of promoter constructs

DNA of each of the four promoter constructs were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, part no. 4303153) was used to carry out the sequencing reactions. Each reaction contained 8µl of Reaction Mix and 1µl of 3.2 µM primer. The volumes of template DNA and H₂O added are as follows: 1.1µl of pYLR110P+luc template and 9.9µl of water; 1.4µl of pYMR251AP+luc template and 9.6µl of water; 2.0-6.0µl of pYMR107P+luc template and 9.0-5.0µl of water; and 0.5-1.5µl of pZEO1P+luc template and 10.5-9.5µl of water.

The thermocycling protocol is described in the ABI protocol, the PCR products were ethanol precipitated by adding 3M NaOAc and absolute Ethanol, standing at room temperature for 15 minutes, centrifuging for 20 minutes and washing with 250µl of 70% ethanol. The precipitated DNA was resuspended in 3µl of loading dye and 2µl of each suspension was analyzed on an PE-ABI 377 automated sequencer.

The following promoter constructs pYLR110P+luc and pYMR251AP+luc were each sequenced using four primers:

YEp13 F2 : CCTCAATTGGATTAGTCTCA - SEQ ID NO:13- aligns to the YEp13 backbone, 290bp 5' of the Hind III site.

Luc R1 : CACCTCGATATGTGCAICTG - SEQ ID NO:14- aligns to the Luc ORF, 150bp 3' of the NdeI site.

Forward PCR primer: forward primer used to PCR clone promoter, i.e., SEQ ID NO:5 and SEQ ID NO: 7.

Reverse PCR primer: reverse primer used to PCR clone promoter, i.e., SEQ ID NO:6 and SEQ ID NO:8.

The remaining promoter constructs (pYMR107P+luc and pZEO1P+luc) were each sequenced using primers Yep13 F2 and Luc R1. Combining the data from all primers completely sequenced the promoter regions and spanned the cloning sites of the original vector.

Deviations from published genomic sequences

All sequences differ by a few base pairs around the ATG, this results from the creation of an NdeI site at the 3' end of the promoter. In addition, the following further alterations from published sequences were identified.

pYLR110P+luc: A substitution of a C for a T had taken place at a base pair 361 of the sequence.

pYMR107P+*luc*: In the initial construct (for which luciferase reporter data is described), a cloning artifact led to the junction between the promoter region and the LUC ORF in pYMR107W+*luc* to have the sequence: CATATATG (where ATG is the luciferase translational start site). This sequence was modified by site directed mutagenesis to create the sequence CATATG, which generates a novel NdeI site at the promoter/luciferase junction. Subsequent luciferase expression analysis confirmed that expression from the NdeI site modified pYMR107P+*luc* construct did not differ significantly from the original construct, therefore the sequence of the corrected CATATG construct is included herein.

Other Modifications

pYMR107P+*luc*: Cloning artifacts created an additional HindIII site and linker to the 5' (i.e. outside) of the pYMR107P+*luc* and promoters:

Instead of:

HindIII *NotI* promoter 5'
AAGCTT-CGCGGCCGCG-NNNNNNN SEQ ID NO:17

The sequence is:

HindIII *HindIII* *NotI* promoter 5'
AAGCTT-AGCT-AAGCTT-CGCGGCCGCG-NNNNNNN SEQ ID NO:18.

EXAMPLE 4

Luciferase assays of promoter activity

Transformation of *S. cerevisiae* with promoter constructs.

S. cerevisiae strain 11C was transformed with five promoter constructs. This strain carries six metabolic markers, Ade, Trp, Ura, Lys, Leu and His. It has the genotype: *ade2-161, trp1-D63, ura3-52, lys2-801, leu2D1 &/or leu2-3 &/or leu2-112, hisD200 &/or hisD200*. 11C was generated by crossing the strains YPH500 (Mat a *ura3-52 lys2-801 ade2-161 trp1-D63 hisD200 leu2D1*) and AH22 (MATa *leu2-3 leu2-112 his4-519 can1*).

11C cells were streaked from a glycerol stock onto a YPD plate and grown at 30°C for two days. The cells were transformed with the five plasmids, pYLR110P+*luc*, pYMR251AP+*luc*, pYMR107P+*luc*, & pZEO1P+*luc* and pPRB1P+*luc* to act as a control.

The transformations were carried out using the Quick and Easy method (Gietz, R.D. and

R.A. Woods, 1994, *Molecular Genetics of Yeast: Practical Approaches* pp. 121-134. 10ml of plasmid was added to the transformation mix in each case. The whole transformation mixes were plated out onto -Leu plates and incubated at 30°C for three days. Three individual colonies from each transformation plate were picked and used to inoculate 10ml YPD cultures. The 10ml cultures were incubated in an orbital shaker set to 200rpm and 30°C. Cells were harvested from the cultures at two points. First, at a point at which the OD

of the culture was close to 1.0, at which time a 4ml sample was taken. Second, a 3ml sample was taken after an incubation time of 45 hours. The ODs and incubation time of each sample is shown in Table 5. For all harvested samples, the cells were immediately spun down at 3000rpm and 4°C, washed in 5ml of dH₂O, repelleted and frozen at -20°C.

measured using the Packard TopCount. The luminescence readings obtained after 0.03min are shown below in counts per second (CPS) in Table 6.

Table 5

Plasmid	Clone number	OD at time of harvesting first 4ml sample	Incubation time at harvesting of first sample (hours)	OD at time of harvesting second 3ml sample
PPRB1P +luc	7	0.98	24.5	4.80
	8	0.68	28	5.56
	9	1.15	28	5.66
PYLR110P +luc	8	1.12	28	5.50
	9	0.46	28	4.38
	10	1.16	24.5	5.51
PYMR231AP +luc	8	1.20	24.5	4.99
	9	1.05	27	4.71
	10	1.15	27	5.18
PYMR107P +luc	1	1.06	27	5.47
	2	0.49	28.5	4.54
	3	0.97	25.5	5.58
PZEO1P +luc	1	1.02	28.5	4.84
	2	0.62	28.5	4.97
	3	0.42	28.5	4.31

Analysis of luciferase activity

All of the samples were analyzed for luciferase activity, using the LucLite

Luciferase Reporter Gene Assay Kit (Packard, cat.no 6016911). The cells were prepared by resuspending in PBS and diluting to a final concentration of 6×10^6 cells/ml. 100ml of each cell suspension was pipetted into wells in duplicate on two 96 well plates, so that each well contained 6×10^5 cells. The plates were incubated at 30°C for 10 minutes. 100ml of a 1 in 2 dilution of reconstituted substrate was added to each well, and the plate was further incubated at room temperature for 10 minutes. The luminescence was then

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Table 6.

Plasmid	Clone number	First sample			Second sample		
		Readings (CPS)	Average	Average	Readings (CPS)	Average	Average
PPRB1P +luc	7	35890	35690	35790	20322	20975	20648
	8	25498	25276	25387	52997	51778	52388
	9	24137	27797	25967	49192	46971	48081
PYLR110P +luc	8	52354	53618	52986	41789	38904	40346
	9	105599	99776	102537	85562	84468	85015
	10	107531	109226	107486	22507	22436	22471
PYMR231AP +luc	8	71993	69797	70895	40869	40702	40536
	9	98853	98389	98621	51159	49828	50493
	10	83210	87546	85378	70091	74576	72334
PYMR107P +luc	1	9046	8650	8848	6790	28505	28505
	2	3996	4009	4002	1945	24391	23915
	3	3018	3236	3127	1069	23866	23408
PZEO1P +luc	1	64137	63162	63649	47469	45769	46619
	2	19579	18329	18954	44910	42982	43946
	3	87572	90317	88944	142414	142262	142338

The results are summarized in Table 7.

Table 7.

Promoter	mRNA levels	Luciferase Expression Glucose	Luciferase Expression Ethanol
PRB1	Ethanol Induced	1.00	1.00
YLRI10C	Highly Ethanol and Glucose Induced	3.03	1.22
YMR231WA	Highly Ethanol and Glucose Induced	2.92	1.35
YMR107W	Ethanol Induced	0.21	0.95
ZE01	Very Highly Ethanol	3.62	2.89

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15 The purified promoter fragment can be ligated into the isolated and purified HindIII, NdeI, double-digested pPRBP1+luc backbone such that the promoter fragment is operably linked to a luciferase gene and transformed into *E. coli*, as described in Example 3. The new expression vector comprising a fragment of YLR110C, YMR251WA, YMR107W, or ZEO1 promoter region can be isolated and purified from *E. coli*, sequenced, and transformed into yeast as described in Example 3.

To analyze promoter activity, luciferase assays as described in Example 4, can be conducted using *S. cerevisiae* cultures that have been transformed with the expression vector comprising a fragment of the YLR110C, YMR251WA, YMR107W, or ZEO1 promoter operably linked to a luciferase gene and *S. cerevisiae* cultures that have been transformed with pPRB1P+luc. The *S. cerevisiae* cultures are grown in medium containing a non-fermentable carbon source, such as ethanol, or a fermentable carbon source, such as glucose, or both. Cells are obtained from the cultures and analyzed for luciferase activity as described in Example 4.

A promoter fragment is operative if it expresses at least 75% of the luciferase activity as the PRB1 promoter. Preferably, an operative promoter fragment expresses at least 100%, 200%, 300%, 400%, or more of the luciferase activity as the PRB1 promoter.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 Polynucleotide sequence of promoter YLR110C
SEQ ID NO:2 Polynucleotide sequence of promoter YMR251WA
15 SEQ ID NO:3 Polynucleotide sequence of promoter YMR107W
SEQ ID NO:4 Polynucleotide sequence of promoter ZEO1
SEQ ID NO:5 Forward PCR primer for YLR110C
SEQ ID NO:6 Reverse PCR primer for YLR110C
SEQ ID NO:7 Forward PCR primer for YMR251WA
20 SEQ ID NO:8 Reverse PCR primer for YMR251WA
SEQ ID NO:9 Forward PCR primer for YMR107W

SEQ ID NO:10 Reverse PCR primer for YMR107W
SEQ ID NO:11 Forward PCR primer for ZEO1
SEQ ID NO:12 Reverse PCR primer for ZEO1
SEQ ID NO:13 Yep13 Forward PCR primer
5 SEQ ID NO:14 Luc R1 Forward PCR primer
SEQ ID NO:15 Primer used in cDNA sequencing
SEQ ID NO:16 Control oligonucleotide used in GeneChip Microarray assay
SEQ ID NO:17 Original pYMR107P+luc sequence
SEQ ID NO:18 Modified pYMR107P+luc sequence
10 SEQ ID NO:19 Nucleotide sequence of pYLR110P+luc
SEQ ID NO:20 Nucleotide sequence of pYMR251AP+luc
SEQ ID NO:21 Nucleotide sequence of pYMR107P+luc
SEQ ID NO:22 Nucleotide sequence of pZEO1P+luc
SEQ ID NO:23 Nucleotide sequence of pYLR110P
15 SEQ ID NO:24 Nucleotide sequence of pYMR251AP
SEQ ID NO:25 Nucleotide sequence of pYMR107P
SEQ ID NO:26 Nucleotide sequence of pZEO1P
SEQ ID NO:27 Nucleotide sequence of pPRB1P
SEQ ID NO:28 Nucleotide sequence of pPRB1P+luc
20 SEQ ID NO:29 YLR110C promoter region
SEQ ID NO:30 YMR251WA promoter region

SEQ ID NO:31 YMR107W promoter region

SEQ ID NO:32 ZEO1 promoter region

CLAIMS

1. A yeast promoter which comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4, wherein the promoter is operative to express a nucleic acid molecule encoding a polypeptide when operably linked to said nucleic acid molecule.
2. The yeast promoter of claim 1, wherein the promoter comprises at least 50 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.
3. The yeast promoter of claim 1, wherein the promoter comprises at least 100 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.
4. The yeast promoter of claim 1, wherein the promoter comprises at least 200 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.
5. The yeast promoter of claim 1, wherein the promoter comprises at least 300 contiguous nucleotides of an isolated and purified polynucleotide selected

from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

6. The yeast promoter of claim 1, wherein the promoter comprises at least 400 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

7. A yeast promoter which comprises an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

8. A yeast promoter fragment which comprises at least 17 contiguous nucleotides of a polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4, wherein the fragment has promoter activity as determined by the steps of:

- (a) cloning the fragment into a yeast expression vector, wherein the fragment is operably linked to a reporter gene;
- (b) transforming yeast cells with the yeast expression vector;
- (c) growing the yeast cells in yeast cell culture under conditions favorable for expression of the reporter gene; and
- (d) assaying the yeast culture for a reporter protein expressed by the reporter gene;

wherein expression of the reporter gene indicates the fragment has promoter activity.

9. A yeast expression vector comprising the yeast promoter of claim 1.
10. The yeast expression vector of claim 9 wherein the yeast expression vector is selected from the group consisting of pYLR110P+luc, pYMR251AP+luc, pYMR107P+luc, pZEO1P+luc, pYLR110P, pYMR251AP, pYMR107P, and pZEO1P.

11. The yeast expression vector of claim 9 wherein activity of the promoter is controlled by varying the level of a non-fermentable carbon source in a medium of yeast cells in culture, wherein the yeast cells are transformed with said yeast expression vector.

12. The yeast expression vector of claim 11 wherein the non-fermentable carbon source is ethanol.

13. A yeast expression vector comprising a yeast promoter which comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4, wherein the promoter is operative to express a nucleic acid molecule encoding a polypeptide when operably linked to said nucleic acid molecule, wherein promoter activity is controlled by varying the level of a fermentable carbon source in a medium of yeast cells in culture, wherein the yeast cells are transformed with said yeast expression vector.

14. The yeast expression vector of claim 13 wherein the fermentable carbon source is glucose.
15. A yeast expression vector comprising a yeast promoter which comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4, wherein the promoter is operative to express a nucleic acid molecule when operably linked to said nucleic acid molecule, wherein promoter activity is controlled by varying the level of a fermentable carbon source and a non-fermentable carbon source in a medium of yeast cells in culture, wherein the yeast cells are transformed with said yeast expression vector.
16. The yeast expression vector of claim 15 wherein the fermentable carbon source is glucose.
17. The yeast expression vector of claim 15 wherein the non-fermentable carbon source is ethanol.
18. A yeast cell transformed with the yeast expression vector of claim 9.
19. A yeast cell transformed with the yeast expression vector of claim 10.
20. A method for producing a polypeptide comprising the steps of:
 - (a) constructing a yeast expression vector wherein a nucleic acid encoding the polypeptide is controlled by the yeast promoter of claim 1;
 - (b) transforming a culture of yeast cells with the yeast expression vector;

- (c) maintaining the yeast cells in culture so that the polypeptide is expressed; and
- (d) recovering the polypeptide.

21. A method for producing a polypeptide comprising the steps of:
 - (a) cloning a nucleic acid molecule encoding the polypeptide into an expression vector selected from the group consisting of pYLR110P+luc, pYMR251AP+luc, pYMR107P+luc, pZEO1P+luc, pYLR110P, pYMR251AP, pYMR107P, and pZEO1P, wherein the nucleic acid molecule is operably linked to a promoter of the expression vector;
 - (b) transforming a culture of yeast cells with the yeast expression vector;
 - (c) maintaining the yeast cells in culture so that the polypeptide is expressed; and
 - (d) recovering the polypeptide.
22. A method for producing a polypeptide comprising the steps of:
 - (a) constructing a yeast expression vector wherein a nucleic acid molecule encoding the polypeptide is controlled by a yeast promoter which comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4;
 - (b) transforming a culture of yeast cells with the yeast expression vector;

- (c) maintaining the yeast cells in culture medium and controlling the expression of the nucleic acid molecule encoding the polypeptide by varying the level of a fermentable carbon source in the culture medium; and
 - (d) recovering the polypeptide.
23. The method of claim 22 wherein the fermentable carbon source is glucose.
24. A method for producing a polypeptide comprising the steps of:
- (a) constructing a yeast expression vector wherein a nucleic acid molecule encoding the polypeptide is controlled by the yeast promoter of claim 1;
 - (b) transforming a culture of yeast cells with the yeast expression vector;
 - (c) maintaining the yeast cells in culture medium and controlling the expression of the nucleic acid molecule encoding the polypeptide by varying the level of a non-fermentable carbon source in the culture medium; and
 - (d) recovering the polypeptide.
25. The method of claim 24 wherein the non-fermentable carbon source is ethanol.
26. A method for producing a polypeptide comprising the steps of:
- (a) constructing a yeast expression vector wherein a nucleic acid molecule encoding the polypeptide is controlled by a yeast promoter which comprises at least 17 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:4;
 - (b) transforming a culture of yeast cells with the yeast expression vector;

- (c) maintaining the yeast cells in culture medium and controlling the expression of the nucleic acid molecule encoding the polypeptide by varying the level of a fermentable carbon source and a non-fermentable carbon source in the culture medium; and
 - (d) recovering the polypeptide.
27. The method of claim 26 wherein the fermentable carbon source is glucose.
28. The method of claim 26 wherein the non-fermentable carbon source is ethanol.
29. A method of identifying a promoter fragment, wherein the fragment has promoter activity comprising the steps of:
- (a) generating a fragment comprising at least 17 contiguous nucleotides of an isolated and purified polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4;
 - (b) cloning the fragment into a yeast expression vector, wherein the fragment is operably linked to a reporter gene;
 - (c) transforming yeast cells with the yeast expression vector;
 - (d) growing the yeast cells in yeast cell culture under conditions favorable for expression of the reporter gene; and
 - (e) assaying the yeast culture for a reporter protein expressed by the reporter gene;

wherein expression of the reporter gene indicates the fragment has

promoter activity.

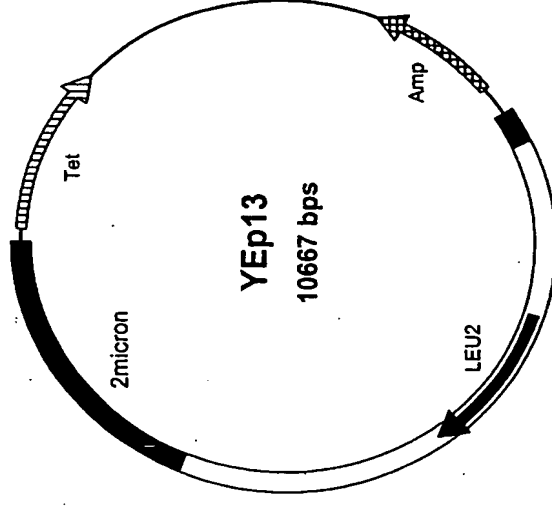


FIGURE 1

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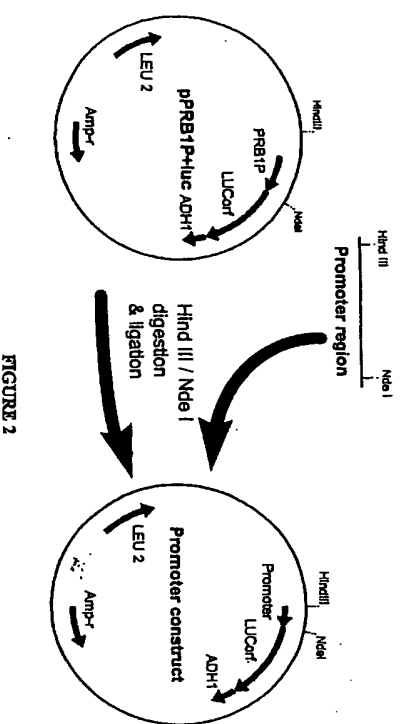


FIGURE 2

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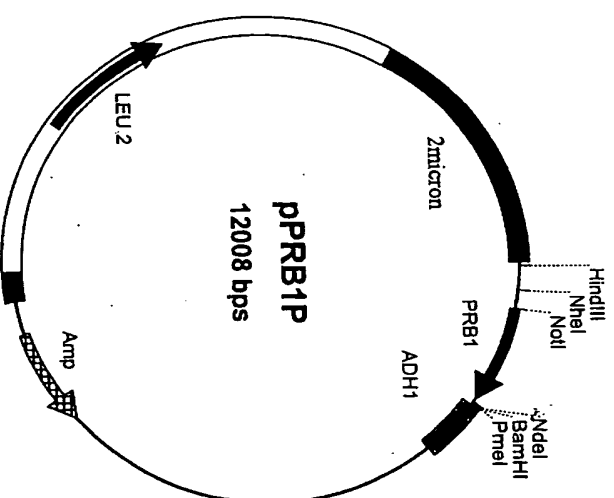


FIGURE 3

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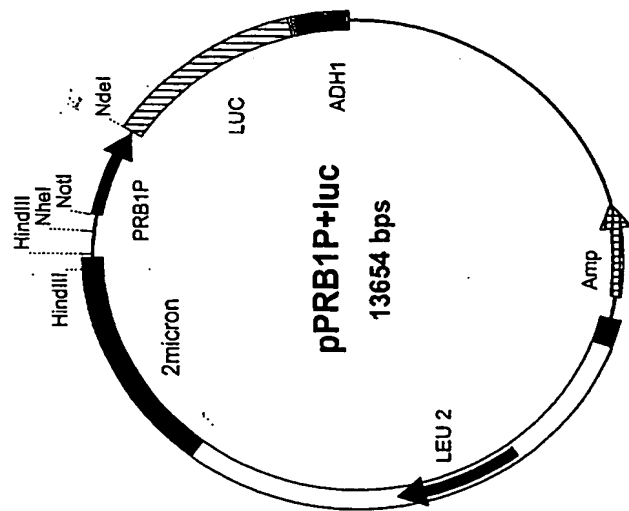


FIGURE 4

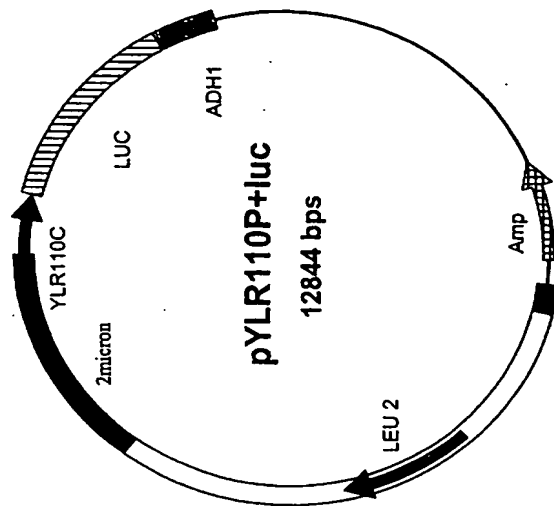


FIGURE 5

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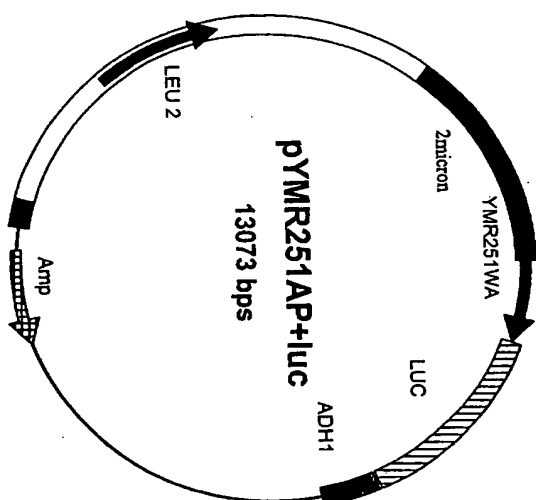


FIGURE 6

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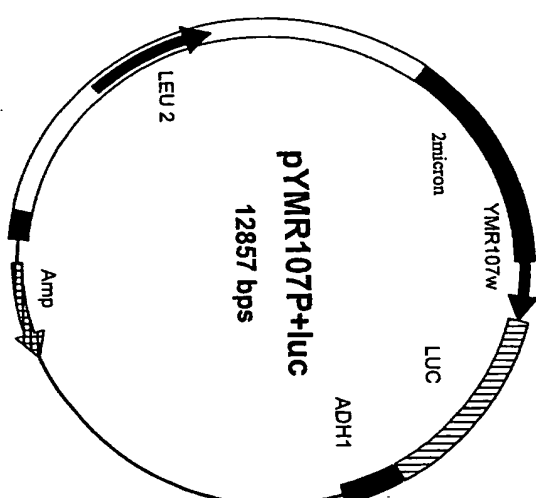


FIGURE 7

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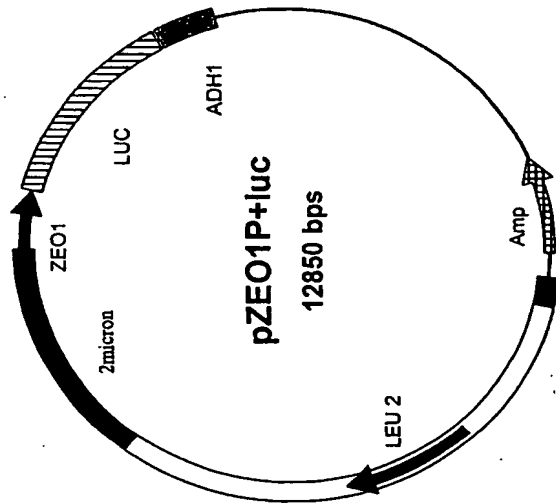


FIGURE 8

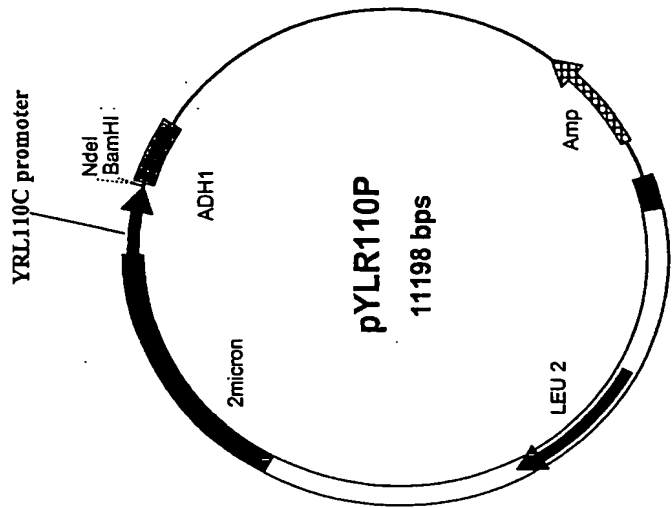


FIGURE 9

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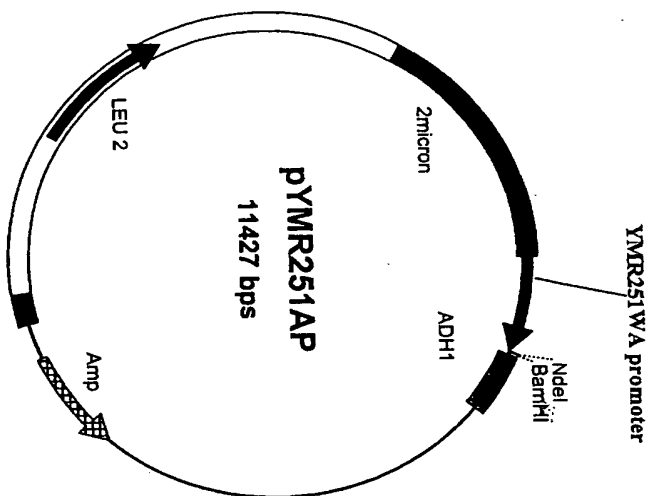


FIGURE 10

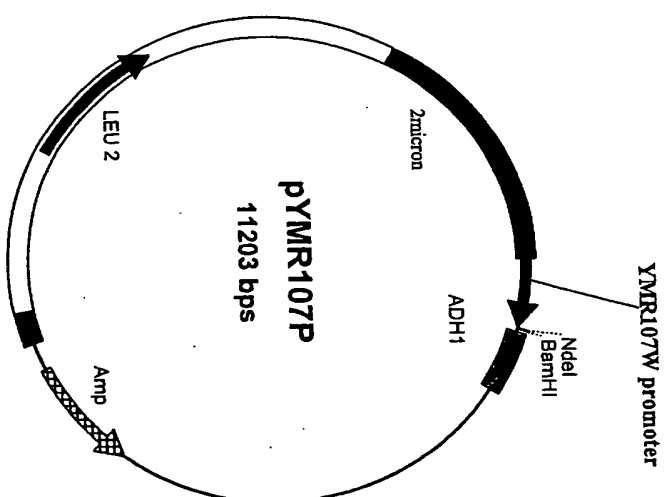


FIGURE 11

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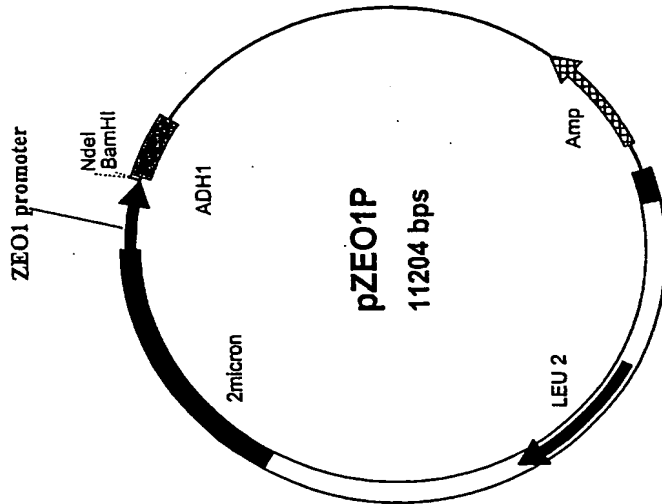


FIGURE 12

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Figure 13 YLR110C promoter region (SEQ ID NO:29)
Sequence shown: Chr XII 370650 to 370051 (reverse orientation)

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1  AGAACCAAT GGGAAATCG GAATGGTCC AGACTGCTT TGAGTGTCTG
   TCTGGTTTA CCGTTTAGC CTACCCAGG TCTTGACAA ACTCAGACC
   ATGCAAGCTTCGGGCGC YLR-F
51  CTATGGCGT CTGATTCCG TTGGGAAT CCTTGGCGG GCGCCCTCT
   GATAACGGCA GACTAAAGG ABAACCTTA GBAACGGCG GCGGGGAGA
101  CBAACTCCG CACAAGTCC AGAAGCGGG AAGAAATAA AACGCCACCA
   GTTTGAGGC GTGTACGGG TCTTTGGCG TTCTTTTAT TTGGGTGGT
151  AAAAAAAAA AATAAAGCC AATCTCGAA GGTGGGTTG TAGGCCCTG
   TTTTTTTT TTATTTTCG TTGGAGCTT GCGCCCGCC ATCGGGACC
201  ATTATCCGT ACGATATTT CTCAGAGTA AAAAAAGGT TTGTTTGG
   TAATAGGCA TGTCTAATA GGTCTCTAT TTTTGGCA AACAAAGCT
251  ATTCGCCAT TCGGGCCAC CTAGCGCGT AACTTGCNA CAACTATCTG
   TAAGGGTAA AGCGCGGTG GATCGCGCA TAGAAGCTT GTTGATAGC
301  CGATACTCA GCAATTTTG CATATTCGT TTGCAATAT GCGATAATG
   GCTATTGAT CTTTAAAC GTATAGCAC AACCTCAAA CGCTATTAC
351  GAGTCTTACT TCCACATAA CGCAGAAAG AATGTGAGA AAATTTTGA
   CTCAGATGA AGTTGTATT GCGTCTTTC TTACACTCT TTAAAACGT
401  TCCTTTGCT CCGTTCAAGT ATATAAGTC GCGATGCTG ATATCTTTC
   AGGAACGGA GCGAGTTCA TATATTGAG CCGTACGAC TATTAGAA
451  TTTCATCTT ACATGTTCT AATATTCTT ATCTCTCTT ATTCTTCT
   AAGGTAGGA TGTACAGA TTAATAGAA TAGAGGAAA TAAGAAAGA
501  AACATACCA GAATTAATC TTCTGTATT CGCTTAAAC CTATATCAT
   TTGTATGGT CTTTAATTG AAGACATGA CGMAATTTG GATATCTTA
   YLR-R
551  AATGCAATTT TCTACTCTG CTCTATCGC GCGTCTGCC GCTGTGCTT
   TTACGTTAA AAGTGCAGC GAGATAGCG GCGACGCGG CCGACGAAA
   A CCGGACC
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YLR11W ORF = Underline

YLR110C ORF = Bold

YLR-F = SEQ ID NO:5

YLR-R = SEQ ID NO:6

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Figure 14 YMR251W/A promoter region (SEQ ID NO:30)

Sequence shown: CHR XIII 773951 TO 774800

```

1  GCCACGGGTC AACCGAATG GATCACGCC ACTGGGGCCC AAGCTGATA
   GGTGGCCGAG TTGGGGTAC CTTAGTGGG TGAACCGGG TTGCGACTAT
   AGCTAAGCTTGGGGCGG YMR-F
51  TCGGACCTGC ATGAATTTT TTTTTCCT TCGATTAGA GCGACACACA
   AGGCTGGAGG TACTTTAAA AAAAAAAGA AGTAACTGT GCGTGTGTGT
101  TCACATAGAC TGGGTGATA AATACACTA GGAATAAC ATAAAGACA
   AGGTATCTTG ACGCAATAT TTTATGTAT GCTTTTGG TATTTCTGT
151  AAGGGATACC TACTTGAAG GAAAGAGAG ACGCTGTGA GGGGATGGG
   TTGCGTATGG ATGAACCTTC CTTTCTGGS TCGAACATC CCCCTACCC
201  GCGTAAAGAG TCATTCACTT TCTTTCCCT TCGCGTCCG GACCGGGAG
   CCGATTCTTC AGTAATGAA AAAAAAGGA AGCGCAGGC CTGGGCGCTG
251  CCGTCTCTTC CCGGACGAT TTCTCCCTT CATATCTTC TTTTATCTT
   GGGAGGAGAG GGGCGTGTCA AAAAAAAGA GTATTAAGG AAAATTAAGA
301  ATCCCGTTGA AGCAACCGCA CTATGACTA ATGTGTCTGG ACATCTCAT
   TAGGGCAACT TCGTTGGCGT GATCTGATT TACCAAGACC TGTAAAGGTA
351  GCGTGTGACT TGTGTGTATC TCACATGTGT AACGGCAGG TGGCTGGAA
   CCGACACTGA ACAACATAG AGTGTACCA TTGCGTGGC ACCGAGCTT
401  ACGTCTCTCT CATTACATTT CTAGAACAG GCGTACAGTC TCGATTAATG
   TCGCAAGGAA GCACTGTAA GATCTTGTCC CGAATGCAG ACGTATATC
451  AATAATAGC GCATTTTGC TAGCGCGCC GCGCGCGCG TTTCGCAATA
   TTATATTTGG CATTAAACG ATCGCGGCGG GCGCGCGGG AAAGGTTAT
501  GGAAGGGCCA GTTTATCGGC GAGCTCTAC TTCTTCCAT TTGGGTAAAC
   CCTCTCGGT CAATAGCCG CTTGAGAGG AAAAAAGTA AACCAATCG
551  CCTTCTCTGT TTTGCGCAG TGGTGTCTG AGCTGCGCC GAGAGACATA
   GGAAGAACA AAAGCGGTG ACACACAGG TCGAAGCGG CCTCTGTAT
601  GTGATTAAGG ATGTACTTT CATTAGAGA ATTAGCAGC GAAAAAAGC
   CACTATTCCT TACTTGAAA GCTACTCTC TAACTGTGG CCTTTTGTG
651  TATGGCTAGC TGGAGTGTG TTTTCAATCA TATAAAGG AGAATATTT
   ATACCGATGG ACCCTCAAC AAAATGTAT AATTTTCC TCTTAAACA
701  GCTCACTATG TGAAGTTTC TGGAGCGCT TACTTTAT TCGAGAGAC
   CGAGTATAG ACTGTCAAG ACCGTGAGA ATTGAATA AGGTCTCTG
751  TATCAATATC TACAGATAT GTCAAAAA AAAAACTA ATATTAATA
   ATAGTTAGT ATGTCTAAA CAGTTTTT TTTTCTAT TATTAATTA
801  ATGAATTAAT CTGAATGTG TGTTCGCG GTCGCTTCA CTGCTTAATG
   TACTTCAATA GATTCACA ACMAAGCGG CAGCGAAT GACCAATCA

```

C

YMR251W ORF = Underline

YMR251WA ORF = Bold

YMR-F = SEQ ID NO:7

YMR-R = SEQ ID NO:8

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Figure 15 YMR107W PROMOTER REGION (SEQ ID NO:31)

Sequence shown: CHR XIII 482463 TO 483063

```

1  AAAAGATCCA TCATTAATTT AAAAAAGTC ATTCGCAAG TTAAATATC
   AGCTAAGCTTGGGGCGG YMR107-F
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101  GTCAATATTT AGTAAATACT AACATATAT GAGAGCGCG GCGTACCCC
151  AACGGAAGAG AGTAACTTC CTGCGCTTG CCAAAACCC ATACATGCGC
201  ATCTGGCTCC TGGCAGGGCG GTTATGAGC ATCAAGCCGC TCCCTTAAT
251  GCTAAAGCT CCAAGAGGA CAATTAGA ATATTGGGG AAAGTACAC
301  AGTCAATTTG CGCTTTATG ACTGGGTCT AAGGTACTAG ATGTAGATA
351  GTGTGACAG AATCAGGGAG ATTAAGAGGA GCAAGGTGGG GTAAATATG
401  GCGATACAA TCTTGCTTGG CTATACACC CCAATCTTGG TAGTAGTAT
451  ATAAATAGA GCCTGCCTTC CTAATGAC TCATAAAT TTTTITGT
501  ACGCACTCT GTAACAGAT AAATTAACC AACTATCGA GATTCATAT
   GATAGCT CATAGGTA MODIFICATION AT
551  ATGGTAACTT TTGGAGAGC ATTCGAGTA TACAGACA AAAAGCAGC
   TACCTTACTTA YMR107-R

```

YMR107W ORF = Bold

YMR107-F = SEQ ID NO:9

YMR107-R = SEQ ID NO:10

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SEQUENCE LISTING

Figure 16 ZE01 PROMOTER REGION (SEQ ID NO:32)

Sequence shown: CHR XV 109746 TO 110346

ZE01-F
AGCTAAGCTTCGGGCCCG
1 TCGAGATGTC TCTCGGCTTA GAGCATATAG TGGCGCAGCT AACTGCGCG
51 GAGATCTCTTCACGACGC CGGTGCGCG CTAGTATGCG CCGGAGGTC
101 CGGATGCGCTA TCCCTAGATT TCGTCGTGCG CCGAGCCGAAA TAGTTARAGC
151 TGTGGTTTAT GGGTGCACCA GGGCTTATC GTGTTTATA TCGATGGCGA
201 TTGTGCGCTC CAGTGTATTT TTGTATATCC AATTAGGTT TCTTACCTAA
251 TTTTATTTT ATCATCTTTA GTTAATGCTG GTTGTCTCTG TTCTGCTGCG
301 TTCTGTGCG GTTCTGCTCT TCTCTGTTT CTTCGTGTG TCCGCCATCG
351 CCGATGGGCT TATATGGGT ATATATATAG AGCGAGTTT TACGTGCGAG
401 ATCATCTCAG TTGCTGTGAT AGCTTTCTTA CTTTATTAAT TCGGTTTAA
451 ACCTCATTAAT ACTTATGTTT TCTTTGATCG GTTTTTTCT CAGTATACTT
501 AATAGTTCAA ATCAAAGAA CATACAAAC TACGTTTATA TCAATTAATA
GCATATAT AGTTAATGTA
551 ATGCTGAAA TCAAAACAA AGCTGAAGCT GCGGCCCGAG ATGTCCACAA
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ZE01 (YOL109W) ORF = Bold
ZE01-F = SEQ ID NO:11
ZE01-R = SEQ ID NO:12

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<213> *Saccharomyces cerevisiae*

<400> 31
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aacacataat gaaggacggcg ggtcaacccc aaagcaaaag agtgaacttc ctggccttgg 180
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agtcagtttg cgcctttatg actgggttct aaggtactag atgttgaaata gtgttgacaag 360
aatcaggggag ataaaggagga gcaaggctgg gtaactgatt gcgataacaa tcttgcttgg 420
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INTERNATIONAL SEARCH REPORT

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SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Character of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOHNSTON, M. et al. "The nucleotide sequence of Saccharomyces cerevisiae chromosome XII". NATURE, 29 May 1997, Vol 387, pages 87-90, see especially page 88, left-hand column, paragraph 3	1-29
	--	
X	VANDEBOL, Micheline et al. "Sequence Analysis of a 44 kb DNA Fragment of Yest Chromosome XV Including the Ty1-H3 Retrotransposon, the suf1(+/-) Frameshift Suppressor Gene for tRNA-Gly, the Yeast Transfer RNA-Thr-1a and a Delta Element". YEAST, 1995, Vol. 11, pages 1069-1075, see entire document	1-29
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☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

- * Special categories of cited documents
- "A" document defining the general state of the art which is not considered to be of particular relevance
 - "E" earlier application or patent has been published on or after the international filing date
 - "L" document which may have priority claiming (or which is cited to establish the publication date of another claim) or other special reason (as specified)
 - "O" document referring to an oral disclosure, use, exhibition or other means
 - "P" document published prior to the international filing date but later than the priority date claimed
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 - "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 - "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/ Swedish Patent Office Box 5055 S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Henrik Nilsson /OGU Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	<p>VERHASSELT, Peter et al. "Sequence Analysis of a 37.6kb Cosmid Clone from the Right Arm of Saccharomyces cerevisiae Chromosome XII, Carrying YAP3, HOG1, SNR6, tRNA-Arg3 and 23 New Open Reading Frames, Among Which Several Homologies to Proteins Involved in Cell Division Control and to Mammalian Growth Factors and other Animal Proteins are Found" YEAST, 1997, Vol. 13, pages 241-250, see especially page 249, right hand-column, paragraph 3</p> <p>--</p>	1-29
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X	<p>EMBL/GenBank/DBJ Databases, HUNT, S. et al: "S. cerevisiae chromosome XIII cosmid 9718" 1995-05-21, Accession No. Z49702</p> <p>--</p>	1-29
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X	<p>EMBL/GenBank/DBJ Databases, HUNT, S. et al: "S. cerevisiae chromosome XIII cosmid 9920" 1995-03-12, Accession No. Z48639</p> <p>-----</p>	1-29
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【包括委任状番号】 0104390

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【書類名】特許請求の範囲

【請求項1】

内燃機関と、
前記内燃機関の吸気バルブの開閉タイミミングを変更可能な開閉タイミミング変更手段と、
吸気バルブの開閉タイミミングが前記内燃機関の圧縮行程における筒内の圧力である筒内
圧縮圧力を通常時よりも低減する選角側の所定タイミミングとなる位置で前記開閉タイミン
グ手段変更手段を固定可能な固定手段と、

前記固定手段の状態を検出する検出手段と、
所定のアイドル条件が成立し前記検出手段によって前記固定手段が前記所定タイミミング
となる位置での固定を解除した状態であると検出されたときには前記所定タイミミングより
も進角した位置で前記内燃機関をアイドル運転する通常時アイドル制御を実行するよう前
記内燃機関と前記開閉タイミミング変更手段とを制御し、所定のアイドル条件が成立し前記
検出手段によって前記固定手段が前記所定タイミミングとなる位置での固定を解除できない
状態であると検出されたときには前記通常時アイドル制御と異なる固定時制御を実行する
よう前記内燃機関を制御する制御手段と、
を備えた内燃機関装置。

【請求項2】

請求項1に記載の内燃機関装置であって、
駆動軸に動力の入出力が可能な電動機と、
前記内燃機関の出力軸と前記駆動軸とに接続され、電力と動力との入出力を伴って該内
燃機関からの動力の少なくとも一部を前記駆動軸に出力可能な電力動力入出力手段と、
前記電動機と前記電力動力入出力手段と電力のやり取りが可能な蓄電手段と、
を備えた内燃機関装置。

【請求項3】

前記電力動力入出力手段は、前記駆動軸と前記内燃機関の出力軸と回転可能な回転軸の
3軸に接続され該3軸のうちのいずれか2軸に入出力される動力に基づいて残余の軸に動
力を入出力する3軸式動力入出力手段と、前記回転軸に動力を入出力可能な発電機と、を
備える手段である、請求項2に記載の内燃機関装置。

【請求項4】

前記制御手段は、前記固定時制御を実行するに際して、前記蓄電手段に電力が蓄電され
るよう前記内燃機関と前記電動機と前記電力動力入出力手段とを制御する蓄電制御を実行
する、請求項2又は3に記載の内燃機関装置。

【請求項5】

前記制御手段は、前記固定時制御を実行するに際して、前記蓄電手段に電力を蓄電させ
る蓄電制御と前記内燃機関の運転停止とを前記蓄電手段の残容量に基づいて切り替えて実
行するよう前記内燃機関と前記電動機と前記電力動力入出力手段とを制御する、請求項2
又は3に記載の内燃機関装置。

【請求項6】

前記制御手段は、前記蓄電制御と前記内燃機関の運転停止とを前記蓄電手段の残容量に
基づいて切り替えて実行するに際して、前記蓄電手段の残容量が所定値未満であるときに
は前記蓄電制御を実行するよう前記内燃機関と前記電動機と前記電力動力入出力手段とを
制御し、前記蓄電手段の残容量が前記所定値以上であるときには前記内燃機関の運転を停
止させる、請求項5に記載の内燃機関装置。

【請求項7】

前記制御手段は、前記固定時制御を実行するに際して、前記内燃機関の運転を停止させ
る、請求項1～3のいずれかに記載の内燃機関装置。

【請求項8】

前記制御手段は、前記固定時制御を実行するに際して、前記通常時アイドル制御よりも
高い回転数で前記内燃機関をアイドル運転するよう前記内燃機関を制御する、請求項1～
3のいずれかに記載の内燃機関装置。

【請求項9】

請求項1～8のいずれかに記載の内燃機関装置を搭載し、車軸が前記駆動軸に連結され
てなる車両。

【請求項10】

内燃機関と、前記内燃機関の吸気バルブの開閉タイミミングを変更可能な開閉タイミミング
変更手段と、吸気バルブの開閉タイミミングが通常時よりもコンプレッション仕事を低減す
る選角側の所定タイミミングとなる位置で前記開閉タイミミング手段変更手段を固定可能な固
定手段とを備えた内燃機関装置の制御方法であって、

所定のアイドル条件が成立し前記固定手段が前記所定タイミミングとなる位置での固定を
解除した状態であるときには前記所定タイミミングよりも進角した位置で前記内燃機関をア
イドル運転する通常時アイドル制御を実行するよう前記内燃機関と前記開閉タイミミング変
更手段とを制御し、所定のアイドル条件が成立し前記固定手段が前記所定タイミミングとな
る位置での固定を解除できない状態であるときには前記通常時アイドル制御と異なる固定
時制御を実行するよう前記内燃機関を制御する、
内燃機関装置の制御方法。

【書類名】明細書

【発明の名称】内燃機関装置、それを搭載した車両及び内燃機関装置の制御方法

【技術分野】

【0001】

本発明は、内燃機関装置、それを搭載した車両及び内燃機関装置の制御方法に関する。

【背景技術】

【0002】

従来、内燃機関装置としては、油圧式のアクチュエータを用いて内燃機関の吸気バルブの開閉タイミングを変更するバルブタイミング可変機構と、このバルブタイミング可変機構の開閉タイミング又は最速角位置で固定可能なロックピンを備えたものが知られている（例えば、特許文献1参照）。この装置では、内燃機関の温度が低いときには最速角位置でバルブタイミング可変機構をロックピンにより固定して圧縮圧を高くして内燃機関を始動することにより冷間時の始動性を向上させ、内燃機関の温度が高いときには最速角位置でバルブタイミング可変機構をロックピンにより固定して圧縮圧を低くして内燃機関を始動することにより始動時の振動を減少させる。そして、内燃機関の始動後は油圧を用いてロックピンによるバルブタイミング可変機構の固定を解除して任意の開閉タイミングで内燃機関の運転を行う。

【特許文献1】特開2000-320356号公報

【発明の開示】

【発明が解決しようとする課題】

【0003】

しかしながら、この特許文献1に記載された内燃機関装置では、バルブタイミング可変機構を駆動する油温が高い場合など油の粘性が低いときには、油圧がうまく作用せず、ロックピンが抜けにくくなるがあった。そして、圧縮圧を低減する速角位置でバルブタイミング可変機構が固定されたまま内燃機関のアイドル運転を実行してしまうと、通常のアイドル運転時に比べ内燃機関から出力される動力が小さくなるなどとして、振動が発生してしまうことがあった。

【0004】

本発明は、このような課題に鑑みなされたものであり、吸気バルブの開閉タイミングを変更可能な内燃機関のアイドル運転において振動の発生を抑制することができる内燃機関装置、それを搭載した車両及び内燃機関装置の制御方法を提供することを目的とする。

【課題を解決するための手段】

【0005】

本発明は、上述の目的を達成するために以下の手段を採った。

【0006】

本発明の内燃機関装置は、

内燃機関と、

前記内燃機関の吸気バルブの開閉タイミングを変更可能な開閉タイミング変更手段と、吸気バルブの開閉タイミングが前記内燃機関の圧縮行程における筒内の圧力である筒内圧縮圧力を通常時よりも低減する速角側の所定タイミングとなる位置で前記開閉タイミング手段変更手段を固定可能な固定手段と、

前記固定手段の状態を検出する検出手段と、

所定のアイドル条件が成立し前記検出手段によって前記固定手段が前記所定タイミングとなる位置での固定を解除した状態であると検出されたときには前記所定タイミングよりも進角した位置で前記内燃機関をアイドル運転する通常時アイドル制御を実行するよう前記内燃機関と前記開閉タイミング変更手段とを制御し、所定のアイドル条件が成立し前記検出手段によって前記固定手段が前記所定タイミングとなる位置での固定を解除できない状態であると検出されたときには前記通常時アイドル制御と異なる固定時制御を実行するよう前記内燃機関を制御する制御手段と、

を備えたものである。

【0007】

この内燃機関装置では、所定のアイドル条件が成立し固定手段が筒内圧縮圧力を通常時よりも低減する速角側の所定タイミングとなる位置での固定を解除した状態であるときにはこの所定タイミングよりも進角した位置で内燃機関をアイドル運転する通常時アイドル制御を実行するよう内燃機関と開閉タイミング変更手段とを制御し、所定のアイドル条件が成立し固定手段が所定タイミングとなる位置での固定を解除できない状態であるときには通常時アイドル制御と異なる固定時制御を実行するよう内燃機関を制御する。ここで、開閉タイミング変更手段の固定を解除可能であるときには速角側の所定タイミングよりも進角させてアイドル運転を実行することにより内燃機関の振動が抑えられるが、固定手段が筒内圧縮圧力を通常時よりも低減した速角側の所定タイミングとなる位置での固定を解除できないときには、圧縮される筒内の気体量が少ないことから内燃機関からアイドル回転数を安定して維持可能な動力を出力できないことがあり、この状態で内燃機関のアイドル運転を行うと、振動が生じることがある。ここでは、固定手段が速角側の所定タイミングとなる位置での固定を解除できない状態で通常時アイドル制御を実行してしまうのを固定時制御を行うことによって回避するのである。したがって、固定手段が速角側の所定タイミングとなる位置での固定を解除できないときに、通常時アイドル制御を実行することにより生じる内燃機関の振動を抑制することができる。

【0008】

本発明の内燃機関装置は、駆動軸に動力の入出力が可能な電動機と、前記内燃機関の出力軸と前記駆動軸とに接続され、電力と動力との入出力を伴って該内燃機関からの動力の少なくとも一部を前記駆動軸に出力可能な電力動力入出力手段と、前記電動機と前記電力動力入出力手段と電力のやり取りが可能な蓄電手段と、を備えたものとしてもよい。このとき、前記電力動力入出力手段は、前記駆動軸と前記内燃機関の出力軸と回転可能な回転軸の3軸に接続され該3軸のうちのいずれか2軸に入出力される動力に基づいて残余の軸に動力を入出力する3軸式動力入出力手段と、前記回転軸に動力を入出力可能な発電機と、を備える手段であるものとしてもよい。

【0009】

本発明の内燃機関装置において、前記制御手段は、前記固定時制御を実行するに際して、前記蓄電手段に電力を蓄電させるよう前記内燃機関と前記電動機と前記電力動力入出力手段とを制御する蓄電制御を実行するものとしてもよい。こうすれば、蓄電制御を利用して電動機や電力動力入出力手段の駆動を伴って内燃機関から出力する動力を高めるため、内燃機関の振動の発生を抑制しやすい。あるいは、前記制御手段は、前記固定時制御を実行するに際して、前記蓄電手段に電力を蓄電させる蓄電制御と前記内燃機関の運転停止とを前記蓄電手段の残容量に基づいて切り替えて実行するよう前記内燃機関と前記電動機と前記電力動力入出力手段とを制御し、前記蓄電手段の残容量が前記所定値以上であるときには前記内燃機関の運転を停止させるものとしてもよい。こうすれば、蓄電手段の残容量が所定値より小さいときには蓄電制御を利用して電動機や電力動力入出力手段の駆動を伴って内燃機関から出力する動力を高めることが可能であり、蓄電手段の残容量が所定値以上であるときにはアイドル条件が成立していても内燃機関を停止するため、蓄電手段の保護を図ると共に内燃機関の振動の発生を抑制することができ。

【0010】

本発明の内燃機関装置において、前記制御手段は、前記固定時制御を実行するに際して、前記内燃機関の運転を停止させるものとしてもよい。こうすれば、開閉タイミング変更手段の速角側の所定タイミングでの固定を解除できないときにはアイドル条件が成立していても内燃機関を停止するため、内燃機関の振動の発生を防止することができる。

【0011】

より制御されている。エンジンECU24は、CPU24aを中心とするマイクロプロセッサとして構成されており、CPU24aの他に処理プログラムを記憶するROM24bと、データを一時的に記憶するRAM24cと、図示しない入力ポートおよび通信ポートとを備える。エンジンECU24には、エンジン22の状態を検出する種々のセンサからの信号、クランクシャフト223の回転位置を検出するクランクポジションセンサ140からのクランクポジションやエンジン22の冷却水の温度を検出する水温センサ142からの冷却水温、燃焼室内に取り付けられた圧力センサ143からの筒内圧力P_{lin}、燃焼室へ吸排気を行なう吸気バルブ128や排気バルブを開閉するカムシャフトの回転位置を検出するカムポジションセンサ144からのカムポジションセンサ124のポジションを検出するスロットルバルブポジションセンサ146からのスロットルポジション、吸気管に取り付けられたエアフロメータ148からのエアフロメータ信号AF、同じく吸気管に取り付けられた温度センサ149からの吸気温、空燃比センサ135aからの空燃比AF、酸素センサ135bからの酸素信号、ペーンポジションセンサ153からのペーンポジションなどが入力ポートを介して入力されている。また、エンジンECU24からは、エンジン22を駆動するための種々の制御信号、例えば、燃料噴射弁126への駆動信号や、スロットルバルブ124のポジションを調節するスロットルモータ136への駆動信号、イグナイタと一体化されたイグニッションコイル138への制御信号、吸気バルブ128の開閉タイミングの変更可変バルブタイミング機構150への制御信号などが出力ポートを介して出力されている。なお、エンジンECU24は、ハイブリッド用電子制御ユニット70と通信しており、ハイブリッド用電子制御ユニット70からの制御信号によりエンジン22を運転制御すると共に必要に応じてエンジン22の運転状態に関するデータを出力する。

[0021]

動力分配統合機構30は、外歯車のサンギヤ31と、このサンギヤ31と同心円上に配置された内歯車のリングギヤ32と、サンギヤ31に噛合すると共にリングギヤ32に噛合する複数のピニオンギヤ33と、複数のピニオンギヤ33を自転かつ公転自在に保持するキャリア34とを備え、サンギヤ31とリングギヤ32とキャリア34とを回転要素として差動作用を行なう遊星歯車機構として構成されている。動力分配統合機構30は、キャリア34にはエンジン22のクランクシャフト26が、サンギヤ31にはモータMG1が、リングギヤ32にはリングギヤ軸32aを介して減速ギヤ35がそれぞれ連結されており、モータMG1が発電機として機能するときにはキャリア34から入力されるエンジン22からの動力をサンギヤ31側とリングギヤ32側にそのギヤ比に応じて分配し、モータMG1が電動機として機能するときにはキャリア34から入力されるエンジン22からの動力とサンギヤ31から入力されるモータMG1からの動力を統合してリングギヤ32側から出力する。リングギヤ32に出力された動力は、リングギヤ軸32aからギヤ機構60およびデフアレンシャルギヤ62を介して、最終的には車両の駆動輪63a、63bに出力される。

[0022]

モータMG1およびモータMG2は、いずれも発電機として駆動することができると共に電動機として駆動できる周知の同期発電電動機として構成されており、インバータ41、42を介してバッテリー50と電力のやりとりを行なう。インバータ41、42とバッテリー50とを接続する電力ライン54は、各インバータ41、42が共用する正極母線および負極母線として構成されており、モータMG1、MG2のいずれかで発電される電力を他のモータで消費することができるようになっている。したがって、バッテリー50は、モータMG1、MG2のいずれかから生じた電力や不足する電力により充電されることになる。なお、モータMG1、MG2により電力収支のバランスをとるものとするれば、バッテリー50は充電されない。モータMG1、MG2は、いずれもモータ用電子制御ユニット(以下、モータECUという)40により駆動制御されている。モータECU40には、モータMG1、MG2を駆動制御するために必要な信号、例えばモータMG1、MG2の回転子の回転位置を検出する回転位置検出センサ43、44からの信号や図示しない電

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流センサにより検出されるモータMG1、MG2に印加される和電流などが入力されており、モータECU40からは、インバータ41、42へのスイッチング制御信号が出力されている。モータECU40は、ハイブリッド用電子制御ユニット70と通信しており、ハイブリッド用電子制御ユニット70からの制御信号によってモータMG1、MG2を駆動制御すると共に必要に応じてモータMG1、MG2の運転状態に関するデータをハイブリッド用電子制御ユニット70に出力する。

[0023]

バッテリー50は、バッテリー用電子制御ユニット(以下、バッテリーECUという)52によって管理されている。バッテリーECU52には、バッテリー50を管理するのに必要な信号、例えば、バッテリー50の端子間に設置された図示しない電圧センサからの端子間電圧、バッテリー50の出力端子に接続された電力ライン54に取り付けられた図示しない電流センサからの充放電電流、バッテリー50に取り付けられた温度センサ51からの電池温度T_bなどが入力されており、必要に応じてバッテリー50の状態に関するデータを通信によりハイブリッド用電子制御ユニット70に出力する。なお、バッテリーECU52では、バッテリー50を管理するために電流センサにより検出された充放電電流の積算値に基づいて残容量(SOC)も演算している。

[0024]

ハイブリッド用電子制御ユニット70は、CPU72を中心とするマイクロプロセッサとして構成されており、CPU72の他に処理プログラムを記憶するROM74と、データを一時的に記憶するRAM76と、図示しない入力ポートおよび通信ポートとを備える。ハイブリッド用電子制御ユニット70には、イグニッションスイッチ80からのイグニッション信号、シフトレバー81の操作位置を検出するシフトポジションセンサ82からのシフトポジションSP、アクセルペダル83の踏み込み量を検出するアクセルペダルポジションセンサ84からのアクセル開度Acc、ブレーキペダル85の踏み込み量を検出するブレーキペダルポジションセンサ86からのブレーキペダルポジションBP、車速センサ88からの車速Vなどが入力ポートを介して入力されている。ハイブリッド用電子制御ユニット70は、前述したように、エンジンECU24やモータECU40、バッテリーECU52と通信ポートを介して接続されており、エンジンECU24やモータECU40、バッテリーECU52と各種制御信号やデータのやりとりを行っている。

[0025]

こうして構成された実施例のハイブリッド自動車20は、運転者によるアクセルペダル83の踏み込み量に対応するアクセル開度Accと車速Vに基づいて駆動軸としてのリングギヤ軸32aに出力すべき要求トルクを計算し、この要求トルクに対応する要求動力がリングギヤ軸32aに出力されるように、エンジン22とモータMG1とモータMG2とがリングギヤ軸32aに出力されるように、エンジン22とモータMG1とモータMG2とが運転制御される。エンジン22とモータMG1とモータMG2の運転制御としては、要求動力に見合う動力がエンジン22から出力されるようにエンジン22を運転制御する。エンジン22から出力される動力のすべてが動力分配統合機構30とモータMG1と共にエンジン22から出力される動力の出力がリングギヤ軸32aに出力されるようモータMG1およびモータMG2を駆動制御するトルク変換運転モードや要求動力とバッテリー50の充放電に必要な電力との和に見合う動力がエンジン22から出力されるようにエンジン22を運転制御すると共にバッテリー50の充放電を伴ってエンジン22から出力される動力の全部またはその一部が動力分配統合機構30とモータMG1とモータMG2とによるトルク変換を伴って要求動力がリングギヤ軸32aに出力されるようモータMG1およびモータMG2を駆動制御する充放電運転モード、エンジン22の運転を停止してモータMG2からの要求動力に見合う動力をリングギヤ軸32aに出力するよう運転制御するモータ運転モードなどがある。

[0026]

ハイブリッド自動車20は、車両に要求される要求パワーなどに基づいてエンジン22の運転・停止を繰り返す間欠運転を行う。このハイブリッド自動車20は、エンジン22を停止する際には、可変バルブタイミング機構150を駆動してロッキングピン154を溝1

残容量SOCが閾値S1未満であるかを判定する(ステップS140)。この閾値S1は、バッテリー50に電力を充電可能か否かを判定する値であり、残容量SOCの最大値の70%や80%などに定めることができる。バッテリー50の残容量SOCが閾値S1未満であると判定されたときには、バッテリー50へ電力を充電可能であるものとみなし、バッテリー50の充電制御を実行し(ステップS150)、このルーチンを終了する。

[0031]

ここでバッテリー充電制御について説明する。このバッテリー充電制御は、エンジン目標回転数Ne*を所定の充電用回転数Nechに設定すると共にエンジン目標トルクTe*を所定の充電用トルクTtechに設定し、この目標回転数Ne*、目標トルクTe*がエンジン22から出力されるようモータMG1の駆動制御を行うのである。ここでは、エンジン22の充電用回転数Nechは、アイドル回転数Neidlよりも高い回転数(例えば1500rpmや2000rpmなど)に設定されている。また、モータMG1の駆動制御は、エンジンECU24がハイブリッド用電子制御ユニット70に充電指令を送信し、この充電指令を受信したハイブリッド用電子制御ユニット70がモータMG1の目標回転数Nm1*とトルク指令Tm1とを設定し、この設定された目標回転数Nm1*とトルク指令Tm1とになるようにインバータ41を制御することにより行われる。ここで、動力分配統合機構30の回転要素における回転数とトルクとの力学的な関係を示す共線図を図8に示す。図中、左のS軸はモータMG1の回転数Nm1であるサンギヤ31の回転数を示し、C軸はエンジン22の回転数Neであるキャリア34の回転数を示し、R軸はモータMG2の回転数Nm2を減速ギヤ35のギヤ比Grで除したリングギヤ32の回転数NmG1を示し、「ρ」は、動力分配統合機構30のギヤ比を示す。図8に示すように、モータMG1を回転数Nm1、トルクTm1で駆動すると、それに応じた電力がバッテリー50に充電される。

[0032]

ここで、アイドル運転ではエンジン22の回転数Neが比較的小さいことや、筒内圧縮圧力を低減する最速角位置でベーン部152bが固定された状態では圧縮行程で圧縮する混合気量が通常時のアイドル回転数Neに比べて少なくエンジン22からトルクを出力しにくくエンジン22の回転数Neが不安定になってしまう。ここでは、アイドル回転数Neidlよりも高い回転数Nechで、且つモータMG1によってエンジン22の回転を押さえ込む方向にトルクをかけてエンジン22からトルクをより出力する状態(発電状態)とするため、振動や騒音を低減することができ、このように、ロックピン154が筒内圧縮圧力を低減する最速角位置でのベーン部152bの固定を解除できずバッテリー50が充電可能であるときには、エンジン22のアイドル条件が成立していてもバッテリー50への充電制御を実行するのである。

[0033]

一方、ステップS140でバッテリー50の残容量SOCが閾値S1以上であると判定されたときには、バッテリー50へ電力を充電できないものとみなし、エンジン22を停止させ(ステップS160)、このルーチンを終了する。ここで、エンジン22の停止は、燃料噴射弁126からの燃料噴射を停止することによりフリクションを利用してエンジン22の回転数Neを低下させる。このように、ロックピン154が筒内圧縮圧力を低減する最速角位置でのベーン部152bの固定を解除できずバッテリー50が充電できないときには、エンジン22のアイドル条件が成立していてもエンジン22を停止するのである。

[0034]

その後、ロックピン154を作動させるアクチュエータの作動油の温度が低下するなどしてロックピン本体154aに油圧がかかるようになると、ロックピン154によるハウジング部152aとベーン部152bとの固定が解除されて可変バルブタイミング機構150が自由に駆動可能となる。そして、アイドル条件が成立したときには、上述したアイドル要求時制御ルーチンのアイドル制御を実行する(ステップS100～S130)。

[0035]

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58に嵌合させることによりベーン部152bを最速角位置で固定させるよう設定されている。また、ハイブリッド自動車20は、エンジン22を始動する際には、ロックピン154によりベーン部152bを最速角位置で固定し圧縮行程の筒内圧縮圧力を通常時よりも低減した状態でモータMG1によりクランキングを行い、エンジン22が完爆したあとに油圧をロックピン本体154aに作用させ溝158への嵌合を解除し、最速角位置よりも進角側の吸気バルブ128の開閉タイミングでエンジン22の運転を行うよう設定されている。このように、エンジン22の始動時には、吸気バルブ128の開閉タイミングで最速角位置とし筒内圧縮圧力を低減するからエンジン22を始動しやすし、ロックピン154によりハウジング部152aとベーン部152bとを固定するからこれらの間の間隔の隙を防止することができる。

[0027]

次に、こうして構成された実施例のハイブリッド自動車20の動作、特にエンジン22のアイドル運転を実行する際の動作について説明する。図7は、エンジンECU24により実行されるアイドル要求時制御ルーチンの一例を示すフローチャートである。このルーチンは、エンジンECU24がハイブリッド用電子制御ユニット70からアイドル指令を受信している間に所定時間毎(例えば数msec毎)に繰り返し実行される。このアイドル指令は、車両に要求される要求パワーP*が所定の閾値Prefよりも小さい場合などエンジンからのパワーの出力が要求されていないときや、エンジン22の暖機時などのアイドル条件が成立したときなどにハイブリッド用電子制御ユニット70から送信される。以下、説明の便宜のため、シフトポジションSPがパーキングポジションにありエンジン22の暖機時について説明する。

[0028]

アイドル要求時制御ルーチンが実行されると、エンジンECU24のCPU24aは、まず、バッテリー50の残容量SOCをバッテリーECU52から入力する(ステップS100)。次に、油圧を用いてロックピン154の嵌合を解除すると共に予め定められたアイドル運転用の開閉タイミングとなるよう可変バルブタイミング機構150を駆動し(ステップS110)、ロックピン154によるハウジング部152aとベーン部152bとの間の固定が解除されているかを判定する(ステップS120)。ここで、アイドル運転用の開閉タイミングとなるベーン部152bの位置は、本実施例では、基準角となる位置(図5参照)の最速角側の位置に設定されている。また、ロックピン154が解除されているか否かの判定は、可変バルブタイミング機構150を駆動したときにベーン部152bの位置が最速角位置から変化したか否かをベーンポジションセンサ153から入力したベーンポジションに基づいて判定することにより行う。このロックピン154は、例えばロックピン154を作動するアクチュエータの油温が高くなることにより作動油の粘性が低くなると油圧がロックピン本体154aに作用しにくくなり溝158から抜けにくくなる。最速角の開閉タイミングで吸気バルブ128が開閉することになる。なお、可変バルブタイミング機構150が既にアイドル運転用の開閉タイミングとなっているときには、ステップS110の処理は省略される。

[0029]

ステップS120でロックピン154による固定が解除されていると判定されたときには、可変バルブタイミング機構150がアイドル運転用の開閉タイミングとなるのを待つて、アイドル制御を実行し(ステップS130)、このルーチンを終了する。ここで、アイドル制御としては、エンジン22の回転数Neが所定のアイドル回転数Neidl(例えば600rpmや1000rpmなど)となるようにスロットルバルブ124のポジションを調節する処理などを行う。

[0030]

一方、ステップS120でロックピン154による固定が解除されていないと判定されたときには、通常のアイドル制御とは異なるロックピン固定時制御を実行する(ステップS140～S160)。具体的には、まず、ステップS100で入力したバッテリー50の

以上詳述した本実施例のハイブリッド自動車20によれば、所定のアイドル条件が成立しロックピン154が筒内圧縮圧力の低い最速角位置での固定を解除した状態であるときは、この最速角位置よりも進角した位置でエンジン22をアイドル運転し、所定のアイドル条件が成立しロックピン154が最速角位置での固定を解除できない状態であり、バッテリー50の残容量SOCが閾値S1未満であるときにはバッテリー50の充電制御を実行するようエンジン22とモータMG1、MG2とを制御し、バッテリー50の残容量SOCが閾値S1以上であるときにはエンジン22を停止させる。ここで、可変バルブタイミング機構150の固定を解除可能であるときには最速角位置よりも進角させてアイドル制御を実行することによりエンジン22の振動が抑えられるが、ロックピン154が筒内圧縮圧力を通常時より低減した最速角位置での固定を解除できないときには、圧縮される筒内の空気が少ないことからエンジン22からアイドル回転数Neidlを安定して維持可能な動力を出力できないことがあり、この状態でエンジン22のアイドル運転を行うと振動が生じてしまう。ここでは、ロックピン154が最速角位置での固定を解除できない状態が通常時アイドル制御を実行してしまうのをロックピン固定時制御を行うことによって回避するのである。このように、可変バルブタイミング機構150の最速角位置での固定を解除できないときには、モータMG1、MG2の駆動を伴ってバッテリー50を充電することによりエンジン22から出力する動力を高めるため、エンジン22の振動の発生を抑制しやすいため、エンジン22の振動の発生を防止することができる。また、バッテリー50の残容量SOCに基づいてバッテリー50の充電制御とエンジン22の停止とを切り替えるため、バッテリー50を十分に保護することができる。また、バッテリー50の充電制御において通常のアイドル回転数Neidlよりも高い充電用回転数Nechでエンジン22を運転するため、筒内圧縮圧力が小さい状態で通常時のエンジン22の回転数Neidlでアイドル制御する場合に比べてトルク変動が小さくなり、エンジン22の振動の発生を抑制しやすい。

[0036]
なお、本発明は上述した実施例に何ら限定されることはなく、本発明の技術的範囲に属する限り種々の態様で実施し得ることはいうまでもない。

[0037]
例えば、上述した実施例では、バッテリー50の残容量SOCに基づいてバッテリー50の充電制御の実行とエンジン22の停止とを切り替えるものとしたが、バッテリー50の充電制御とエンジン22の停止との少なくとも一方を実行するものとしてもよい。こうしても、エンジン22のアイドル運転において振動の発生を抑制することはできる。

[0038]
上述した実施例では、バッテリー50の残容量SOCに基づいてバッテリー50の充電制御とエンジン22の停止とを切り替えるものとしたが、このバッテリー50の残容量SOCを温度センサ51による電池温度Tbを加味したものに基いてバッテリー50の充電制御とエンジン22の停止とを切り替えるものとしてもよい。こうすれば、バッテリー50の温度状態を用いてバッテリー50の保護を一層図ることができる。

[0039]
上述した実施例では、エンジン22の目標回転数Ne*をエンジン22のアイドル回転数Neidlよりも高い回転数Nechとしてバッテリー50の充電制御を実行するものとしたが、エンジン22の目標回転数Ne*をエンジン22のアイドル回転数Neidlとして充電制御を実行するものとしてもよい。こうしても、エンジン22から動力が出力されることによって、エンジン22の振動を低減することができる。

[0040]
上述した実施例では、アイドル条件が成立しロックピン154が最速角位置での固定を解除できない状態である場合には、バッテリー50の充電制御とエンジン22の停止とを実行するものとしたが、これに代えて、通常時のアイドル制御のエンジン22の回転数Neidlよりも高い回転数でエンジン22をアイドル制御するものとしてもよい。こうすれば、筒内圧縮圧力が小さい状態で通常時のエンジン22の回転数Neidlでアイドル制

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御する場合に比べてトルク変動が小さくなるため、エンジン22の振動の発生を抑制することができる。このとき、エンジン22の回転数Neをアイドル回転数Neidlとするのに必要なアイドル制御量(スロットルバルブ124の開度など)を学習するアイドル学習を行わないものとしてもよい。こうすれば、アイドル制御値が通常と異なる状態で運転されている状態のものになってしまうのを防止することができる。

[0041]
上述した実施例では、最速角位置で可変バルブタイミング機構150をロックピン154により固定するものとしたが、最速角位置から数度進角した位置などで可変バルブタイミング機構150をロックピン154により固定するものとしてもよい。

[0042]
上述した実施例では、ベーン部152bの位置を検出するベーンポジションセンサ153によりロックピン154による可変バルブタイミング機構150の固定が解除されたか否かを検出するものとしたが、ロックピン本体154aの位置を検出するロックピンポジションセンサを可変バルブタイミング機構150に設け、このセンサの出力値を用いてロックピン154による可変バルブタイミング機構150の固定が解除されたか否かを検出するものとしてもよい。このとき、上述のアイドル要求時制御ルーチンにおいて、ロックピン154の固定が解除されたか否かを判定したのに可変バルブタイミング機構150の駆動を行うようにしてもよい。

[0043]
上述した実施例では、ハイブリッド自動車20が停車状態でありエンジン22の暖気時にアイドル条件が成立した場合について説明したが、ハイブリッド自動車20が走行中のときにアイドル条件が成立してもよい。このとき、ハイブリッド用電子制御ユニット70は、駆動軸としてのリングギヤ軸32aに要求される車両の走行用の要求トルクTr*に、モータMG1から出力された反力トルクである $(-1/\rho \cdot Tm1)$ のトルクを加味したトルクがモータMG2から出力されるようインバータ42などを制御すればよい。

[0044]
上述した実施例では、モータMG1、MG2を備えたハイブリッド自動車20としたが、吸気バルブの開閉タイミングが筒内圧縮圧力を通常時よりも低減する連角側のタイミングとなる位置で固定するロックピン154を有する可変バルブタイミング機構150を備えたエンジン22を搭載した車両であれば特にこれに限定されず、例えば、エンジンを始動停止するアイドルストップ自動車に適用するものとしてもよい。この場合においても、上述した実施例と同様に、アイドル条件が成立しロックピン154が最速角位置での固定を解除できない状態である場合には、エンジン22を停止するものとしてもよい。こうすれば、アイドル条件が成立していてもエンジン22を停止するため、エンジン22の振動の発生を防止することができる。

[0045]
実施例のハイブリッド自動車20では、モータMG2の動力を減速ギヤ35により変速してリングギヤ軸32aに出力するものとしたが、図9の変形例のハイブリッド自動車120に例示するように、モータMG2の動力をリングギヤ軸32aが接続された車軸(駆動輪63a、63bが接続された車軸)とは異なる車軸(図9における車輪64a、64bに接続された車軸)に接続するものとしてもよい。

[0046]
実施例のハイブリッド自動車20では、エンジン22の動力を動力分配統合機構30を介して駆動輪63a、63bに接続された駆動軸としてのリングギヤ軸32aに出力するものとしたが、図10の変形例のハイブリッド自動車220に例示するように、エンジン22のクラフトラフト26に接続されたインナーロータ232と駆動輪63a、63bに動力を出力する駆動軸に接続されたアウトロータ234とを有し、エンジン22の動力の一部を駆動軸に伝達すると共に残余の動力を電力に変換する対ロータ電動機230を備えるものとしてもよい。

[0047]

上述した実施例では、エンジン22とモータMG1、MG2とを備えたシリーズ・パレレルハイブリッド自動車としたが、シリーズハイブリッド自動車としてもよいし、パレレルハイブリッド自動車としてもよい。また、こうしたハイブリッド自動車に適用するものに限定されるものではなく、自動車以外の車両や船舶、航空機などの移動体に搭載される内燃機関装置の形態としても構わない。さらに、こうした内燃機関装置の制御方法の形態としてもよい。

【図面の簡単な説明】

【0048】

【図1】本発明の一実施例であるハイブリッド自動車20の構成の概略を示す構成図である。

【図2】エンジン22の構成の概略を示す構成図である。

【図3】可変バルブタイミング機構150の外観構成を示す外観構成図である。

【図4】可変バルブタイミング機構150の構成の概略を示す構成図である。

【図5】インテークカムシャフト129の角度を進角させたときの吸気バルブ128の開閉タイミングおよびインテークカムシャフト129の角度を遅角させたときの吸気バルブ128の開閉タイミングの一例を示す説明図である。

【図6】ロックピン154の構成の概略を示す構成図である。

【図7】実施例のエンジンECU24により実行されるアイドル要求時制御ルーチンの一例を示すフローチャートである。

【図8】動力分配統合機構30の回転要素を力学的に説明するための共線図の一例を示す説明図である。

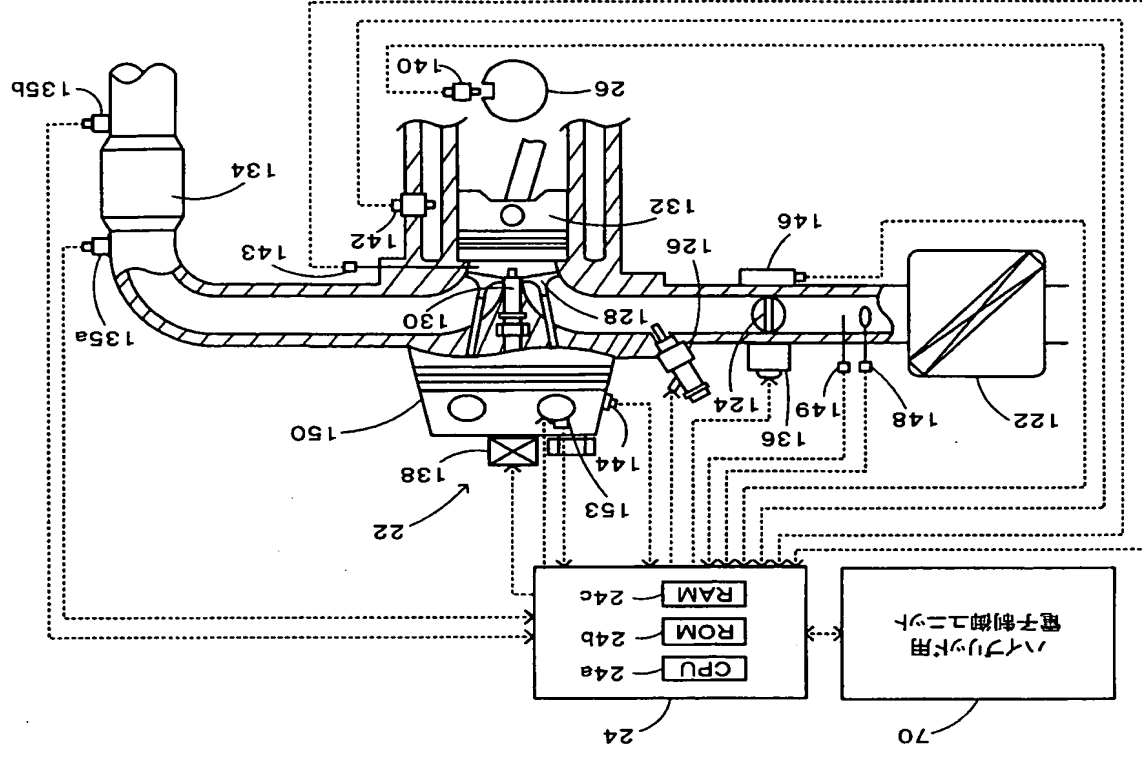
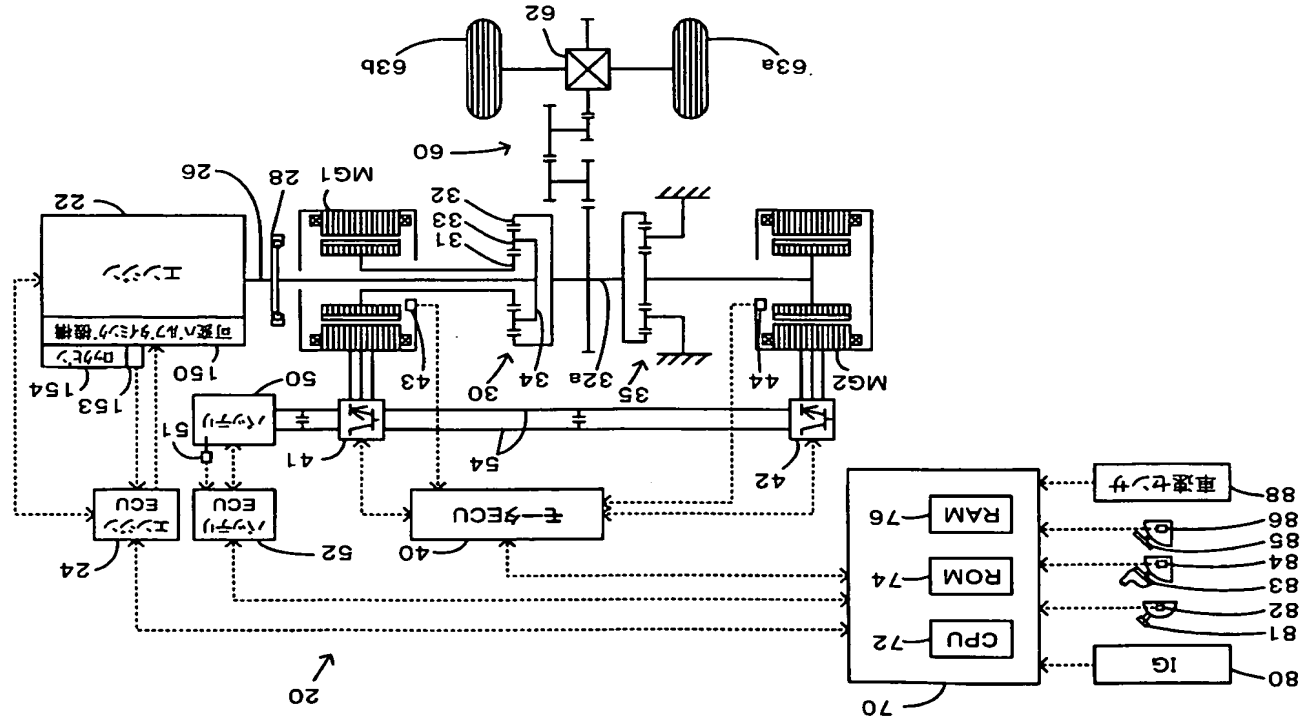
【図9】変形例のハイブリッド自動車120の構成の概略を示す構成図である。

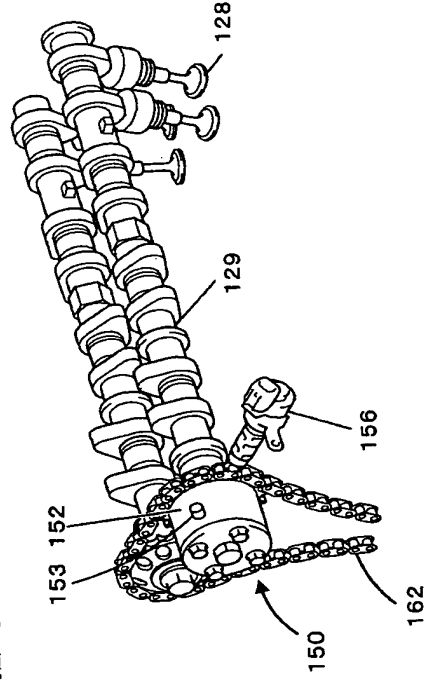
【図10】変形例のハイブリッド自動車220の構成の概略を示す構成図である。

【符号の説明】

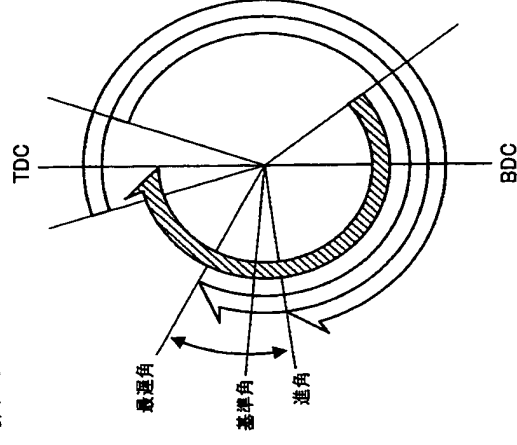
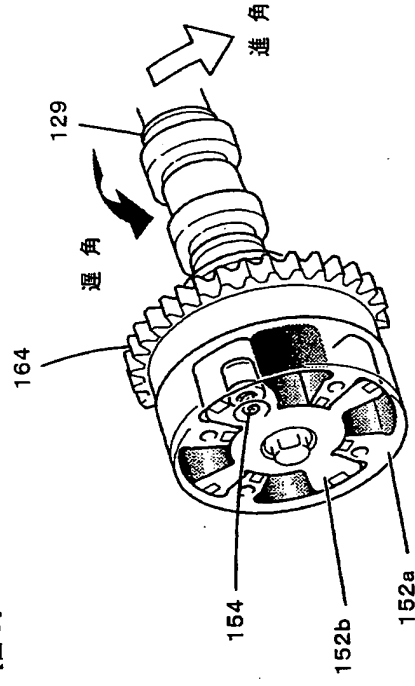
【0049】

20, 120, 220 ハイブリッド自動車、22 エンジン、24 エンジン用電子制御ユニット(エンジンECU)、24a CPU、24b ROM、24c RAM、26 クランクシャフト、28 ダンパ、30 動力分配統合機構、31 サンギヤ、32 リングギヤ、32a リングギヤ軸、33 ピニオンギヤ、34 キャリア、35 減速ギヤ、40 モータ用電子制御ユニット(モータECU)、41, 42 インバータ、43, 44 回転位置検出センサ、50 バッテリ、51 温度センサ、52 バッテリ用電子制御ユニット(バッテリECU)、54 電カライン、60 ギヤ機構、62 デファレンシャルギヤ、63a, 63b 駆動輪、64a, 64b 車輪、70 ハイブリッド用電子制御ユニット、72 CPU、74 ROM、76 RAM、80 イグニッションスイッチ、81 シフトレバー、82 シフトポジションセンサ、83 アクセルペダル、84 アクセルペダルポジションセンサ、85 プレーキペダル、86 プレーキペダルポジションセンサ、88 車速センサ、122 エアクリーナ、124 スロットルバルブ、126 燃料噴射弁、128 吸気バルブ、129 インテークカムシャフト、130 点火プラグ、132 ピストン、134 浄化装置、135a 空燃比センサ、136 スロットルモータ、138 イグニッションコイル、140 クランクポジションセンサ、142 水温センサ、143 圧力センサ、144 カムポジションセンサ、146 スロットルバルブポジションセンサ、148 エアフロメータ、149 温度センサ、150 可変バルブタイミング機構、152 VVTコントロール、154 2a ハウジング部、152b ベーン部、153 ベーンポジションセンサ、154 ロックピン、154a ロックピン本体、154b スプリング、156 オイルコントロールバルブ、158 溝、159 油路、162 タイミングチェーン、164 タイミングギヤ、230 対ロータ電動機、232 インナーロータ 234 アウターロータ、MG1, MG2 モータ。

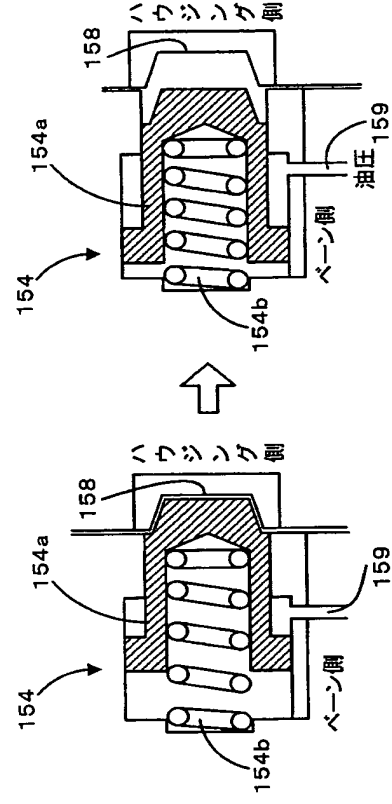




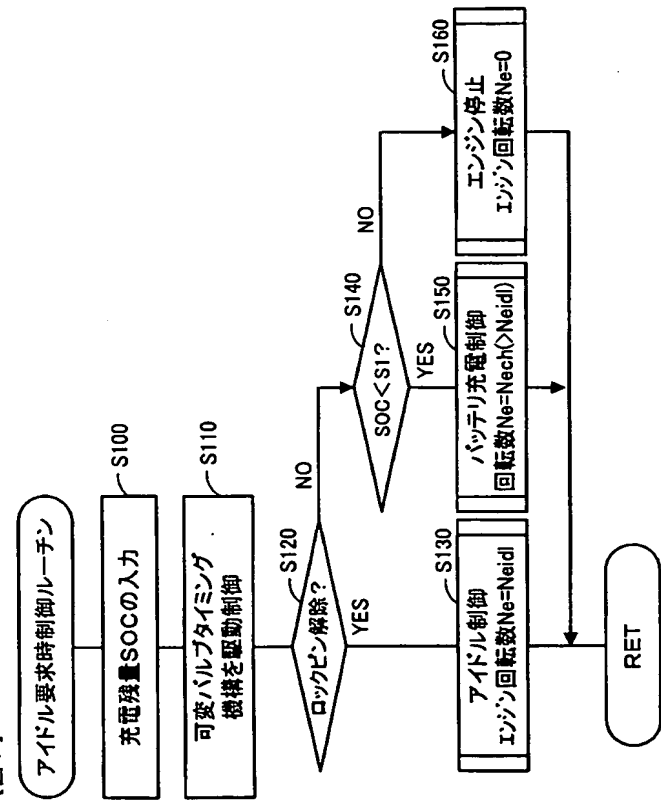
【4】



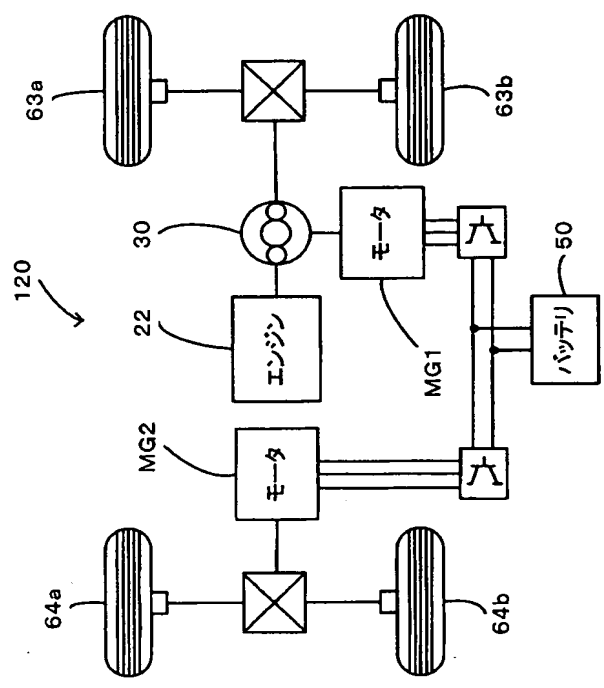
【図6】



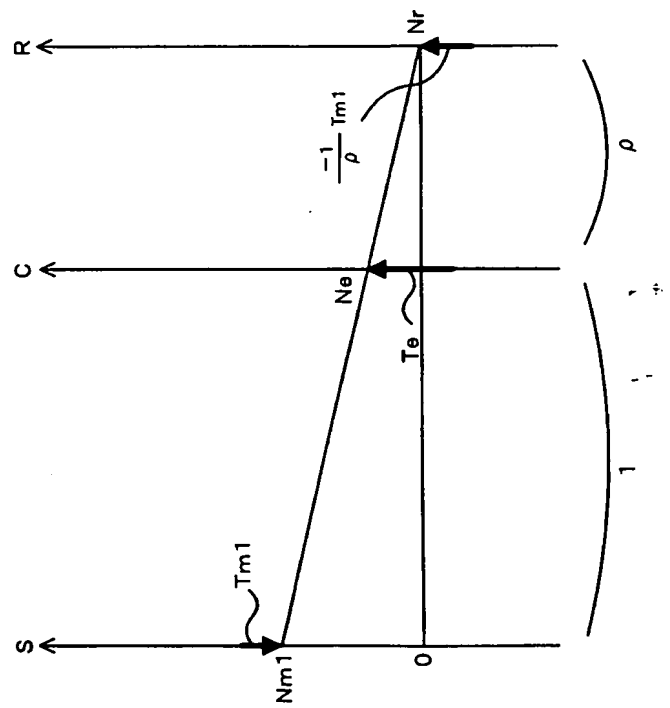
【図7】



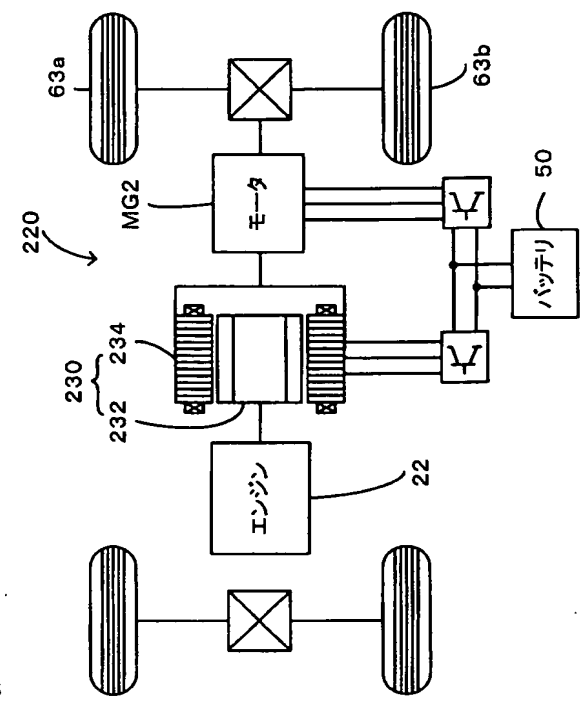
【図9】



【図8】



【図10】



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